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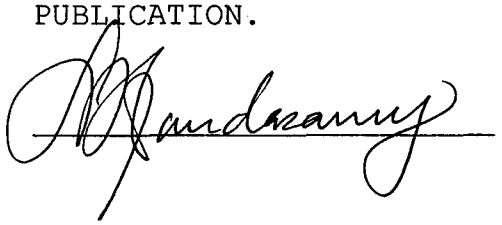

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13. ABSTRACT ***(Maximum 200 Words)***

Thrombospondin-1 (TSP1) is an extracellular matrix glycoprotein that modulates endothelial cell growth, motility, and adhesion. Its role in regulating angiogenesis of breast tumors was examined using site specific mutagenesis, recombinant fragments, and peptide mimetics based on TSP1. Peptides from the type I repeats of TSP1 reproduced the growth inhibitory activity of the intact protein. Stable analogues of the L-forward peptides and of D-reverse or retro-inverso peptide mimetics were prepared that inhibited endothelial cell proliferation stimulated by basic fibroblast growth factor (FGF-2) and selectively induced apoptosis of endothelial cells. The $\alpha 3\beta 1$ integrin was identified as receptor on breast carcinoma cells that mediates adhesive and migratory responses of the cells to TSP1. Over expression of TSP1 in breast carcinoma cells suppresses tumorigenesis and angiogenesis in murine xenografts. The role of the TGFβ-activating and FGF2 antagonist type I repeat sequences in this activity was examined using site-directed mutagenesis of phenylalanine and tryptophan residues required for activity of peptides derived from TSP1. Stably transfected breast carcinoma cell lines expressing these mutants were characterized for tumorigenic potential *in vivo* and behavior *in vitro*.

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Paul J. Roberts 10/28/88

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TABLE OF CONTENTS

SF298 Report Documentation Page		2
Foreword		3
Table of Contents		4
Introduction		5
Body of Report		7
	Task 1	7
	Task 2	10
	Task 3	11
	Task 4	18
Key Research Accomplishments		25
Reportable Outcomes		26
Conclusions		28
Literature Cited		32
Appendices		
A-J	Publications	36
K	Tables	136
L	Figures and Figure legends	139
M	Bibliography of Publications and Meeting Abstracts	160
N	Personnel	162

INTRODUCTION

Growth of many solid tumors is strongly dependent on recruitment of neovascularization. Increased vascularization of primary breast tumors has been associated with increased rates of metastasis to lymph nodes and poorer prognosis (1, 2). Regulation of angiogenesis involves both stimulatory or angiogenic factors and inhibitory or anti-angiogenic factors (3, 4). In normal adult endothelium, high expression of anti-angiogenic factors and limited availability of angiogenic factors maintains the endothelium in a nonproliferative state. Pathological states including wound repair, diabetic retinopathy, or tumor growth may alter the balance of these simulators or inhibitors to allow neovascularization to proceed (3).

Several anti-angiogenic factors have also been identified, including thrombospondins-1 and 2 (5-9). The hypothesis that thrombospondin-1 (TSP1) can inhibit neovascularization of tumors has been confirmed by transfection studies in several tumor models (10-13), reviewed in Appendix A). Synthetic peptides from the type I repeats and recombinant amino-terminal heparin-binding domain from TSP1 mimic the inhibitory activities of intact TSP1 on endothelial cell proliferation and motility (8, 9). These fragments and peptides act at least in part by competing with basic fibroblast growth factor (FGF2) for binding to heparan sulfate proteoglycan receptors on the endothelial cells, which are essential for presentation of FGF2 to its signaling receptor.

The mechanism by which TSP1 inhibits angiogenesis is under active investigation. TSP1 inhibits proliferation and spontaneous tube formation by endothelial cells *in vitro* (14) and inhibits angiogenesis *in vivo* (5, 15). However, endothelial cell responses to TSP1 are complex; the magnitude and direction of the responses depend upon the presence of additional matrix components and growth factors. Immobilized TSP1 promotes endothelial cell adhesion on some substrates (6) but inhibits adhesion on others, including substrates coated with fibronectin (16). Inhibition of adhesion to fibronectin is associated with disruption of focal adhesion contacts (17). TSP1 promotes migration of endothelial cells in chemotaxis and haptotaxis assays but inhibits chemotaxis induced by FGF2 (6).

To understand the mechanisms of these diverse and apparently conflicting effects of TSP1 on endothelial cell behavior, it is necessary to define the domains of TSP1 that interact with the cells, the identity of the endothelial cell receptors that interact with TSP1, and the intracellular responses in transduction and integration of the signals resulting from TSP1 binding to each receptor. Based on inhibition by monoclonal antibodies and sulfated polysaccharides, the heparin-binding domain at the amino-terminus of TSP1 may be responsible for regulation of endothelial proliferation (6). However, a 140 kDa fragment of TSP1 that lacks the amino-terminal region also suppresses endothelial cell growth (5). Thus, multiple sites on the TSP1 molecule may modulate endothelial cell growth and motility. Moreover, based on studies by Murphy-Ullrich et al. (18), inhibition of bovine endothelial cell growth by TSP1 is at least partly due to the inhibitory activity of transforming growth factor β, which complexes with TSP1 and contaminates most TSP1 preparations. We have previously identified two parts of TSP1 that have antiproliferative activity in isolation (8, 9). Recombinant amino-terminal domain inhibited endothelial growth and motility induced by serum or FGF2. Synthetic peptides from the type I repeats also inhibited proliferation to FGF2 and showed a biphasic effect on motility of endothelial cells in the presence of FGF2

that mimicked the activity of intact thrombospondin. Tolsma et al (19) reported that additional peptides from the type I repeats have antiangiogenic activity and identified a sequence in the procollagen domain with antiangiogenic activity. Thus, at least three isolated regions of TSP1 have anti-angiogenic activities, and some of these activities are expressed in synthetic or recombinant constructs without contaminating transforming growth factor-β (TGFβ).

Synthetic peptides from TSP1 were used to further define the activity of the type I repeats (20, 21). The type I peptides of TSP1 define a new class of heparin-binding peptides, since they lack any previously identified heparin-binding consensus sequences and most do not contain any basic amino acids. Studies with homologous peptides showed that two Trp residues and the Ser residue are essential (21). The Trp residues must be spaced less than four residues apart. The Pro residue is essential for proper conformation and activity of the pentapeptide Trp-Ser-Pro-Trp-Ser, but some larger peptides with substitutions of the Pro residue are active.

The synthetic peptides from the type I repeats are especially promising for development of new therapeutics to prevent tumor invasion and metastasis, since they are active *in vitro* at relatively low concentrations. Free peptides, however, often have short half lives in circulation. They are subject to rapid clearance due to their small size and susceptibility to proteolytic degradation.

In several cases, use of polymer conjugates of peptides from extracellular matrix proteins has overcome these limitations (22-24). The peptides from the type 1 repeats of thrombospondin have therefore been conjugated to a ficoll (polysucrose) carrier to increase their stability *in vivo*. We have characterized polysucrose conjugates as proposed for Task 2 of our Statement of Work. Results describing their effects on breast carcinoma and endothelial cells *in vitro* and lack of antitumor activity *in vivo* for breast tumor xenografts have been published (25, 26).

Preparation of retro-inverso or D-reverse analogs is a second method to increase *in vivo* activity of peptides. These analogs have been successfully applied to increase the stability and biological activity of peptide sequences for therapeutic applications (reviewed in (27). Of particular relevance to the TSP1 peptides, an all D-amino acid peptide analog of a peptide from the A chain of the extracellular matrix protein laminin replicated the activity of the natural sequence to influence tumor cell adhesion and growth *in vitro* and *in vivo* (28). The retro-inverso analog of the TSP1 type 1 peptide sequence KRFKQDGGWSHWSPWSSC was chosen as the starting point for preparation of retro inverso analogs. Work proposed in Tasks 1 and 2 resulted in two publications demonstrating the activities of these retro-inverso peptides *in vitro* and *in vivo* to inhibit breast carcinoma and endothelial cell growth (25, 26).

Our second major goal was to define the function of these sequences in the intact TSP1 protein. These studies employed expression of recombinant TSP1 containing site-specific mutations. Stable transfectants of a human breast carcinoma cell line expressing some of these mutants were used to produce the recombinant proteins for *in vitro* characterization. The same cell lines were simultaneously tested *in vivo* for tumorigenic, angiogenic, and metastatic phenotypes. Correlations between these assays provided insight into the role of specific sequences in TSP1 in regulating tumor behavior. Preparation of the mutants for Task 3 has been completed, and stable transfectants have been prepared where

possible. This final report summarizes the characterization of the effects of these mutant thrombospondins on breast and endothelial cells as described in Task 3 and Task 4. Because several of the constructs could not be expressed in stable transfectants, we have modified our experimental plan to utilize transient expression assays to examine their biological activities.

In the course of these experiments, we have also discovered that breast carcinoma cells preferentially use the $\alpha 3\beta 1$ integrin to mediate adhesion and chemotactic responses to TSP1. The activity of this thrombospondin receptor was found to be specifically induced by insulin-like growth factor receptor ligands. Recently, we have also identified the specific sequence in TSP1 that is recognized by the $\alpha 3\beta 1$ integrin and discovered that this integrin plays important roles in the interactions of TSP1 with both the breast carcinoma cells and endothelial cells. We found that the $\alpha 3\beta 1$-binding sequence in TSP1 has a pro-angiogenic activity and discovered that peptides that block this interaction can also be used to inhibit angiogenesis. These new data are also summarized in this report.

BODY OF REPORT:

TASK 1: Preparation of stable analogs of thrombospondin-1 peptides.

METHODS

Materials-- TSP1 was purified from the supernatant of thrombin-stimulated human platelets (29). TSP1 and its fragments were iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) or Bolton-Hunter reagent (Dupont NEN) as previously described (29). Antibodies to native and denatured TSP1 were prepared by immunization of rabbits with native TSP1 or reduced and carboxymethylated TSP1, respectively.

Preparation of synthetic peptides-- The peptides used in this study were synthesized on a Biosearch Model 9600 peptide synthesizer using standard Merrifield solid phase synthesis protocols and t-butoxycarbonyl chemistry (21). Where noted, peptides were also synthesized using fmoc chemistry. Peptides were analyzed by reverse phase HPLC chromatography or gel permeation using a Superdex 75 HR 10/30 column eluted in 0.1 M ammonium acetate, pH 6. Peptides for biological assays were further purified by dialysis using Spectrapor 500 molecular weight cutoff tubing, gel permeation chromatography, or reverse phase purification using C_{18} Sep-pak cartridges. Identities of some peptides were verified by MALDI time of flight mass spectrometry.

Bioassay for inhibition of endothelial and breast carcinoma cell proliferation– Proliferation of bovine aortic endothelial cells was determined as previously described (30). Similar assays were performed using MDA MB 435 human breast carcinoma cells except that the growth medium for the proliferation assays contained 5% fetal bovine serum in RPMI 1640 medium. Apoptosis of the cells exposed to peptides was quantified by electrophoretic analysis of DNA fragmentation or using a DNA fragment ELISA (Boehringer Mannheim) after labeling the cells with bromodeoxyuridine and exposure to the peptides for 24 h.

Tumorigenesis assay in nude mice-- NIH Nu/Nu mice or NIH Beige XID mice, approximately eight weeks of age were injected with 10^5 MDA MB 435 cells by the mammary fat pad route. Wild type MDA cells were used for peptide studies; stable transfectants expressing full length wild type or mutant TSP1 were used to examine the

effects of site-directed mutations in TSP1 on tumorigenesis. Mice were anesthetized with 150-200 µl i.p. of a 1:80 dilution in PBS of a solution containing 25 g. tribromoethanol in 12.5 ml tertiary amyl alcohol. The mammary fat pad was cleaned with ethanol and a 10 mm incision was made directly above the site of injection. Using a 0.1 ml Hamilton syringe and 27 gauge ½" needle, 10 µl of cell suspension, 1 x 10^5 cells in Hanks balanced salts solution for nu/nu mice or 4 x 10^5 for Beige mice, were injected into the fat pad. The incision was closed using 1-2 Autoclips (9 mm, Clay Adams). Autoclips were removed 7 days post-injection.

8-10 animals are injected for each condition, per experiment. Animals were ear punched after injection for subsequent identification. Beginning at day 25 and continuing every day until day 50, the experimental animals for peptide treatment were injected i.v. (tail vein) 100 µl of the free peptide or ficoll conjugates. Animals implanted with transfected MDA cell lines were not treated. Primary tumor size was determined twice weekly by length x width x height measurement, and the animals were observed daily for general health. When the primary tumor of any animal exceeded 20 mm in any dimension, all of the animals were sacrificed. The presence of metastases was determined by gross autopsy and examination of H & E stained sections of step sections of the lungs and draining lymph nodes. The primary tumors were removed, stripped free of other tissues, and weighed. At any time during the experiment, animals suspected of being in distress were sacrificed and examined as above.

RESULTS

We have completed and published the studies based on specific aim 1, to define structural elements responsible for activity of the TSP1 type 1 peptides and to prepare stable analogs with *in vivo* activity (31), Appendix B). We have also completed and published a study of the apoptosis response to the peptides and shown that native TSP1 has the same activity (25), Appendix C). We have also discovered that breast carcinoma cells preferentially use the $\alpha 3\beta 1$ integrin to spread on TSP1 and that the activity of this integrin in breast carcinoma cells is regulated by insulin-like growth factor I and CD98 . This contrasts with endothelial cells, which have been previously demonstrated to use the $\alpha v\beta 3$ integrin as a TSP1 receptor (6, 32). Use of different integrins may account for some of the differential responses of breast carcinoma and endothelial cells to TSP1 and the TSP1 mutants. A manuscript based on these results is attached. To overcome the limitations in using full length recombinant thrombospondins to characterize functions of the type I repeats, we have used truncated recombinant thrombospondins to continue mapping the functional domains for its interactions with the endothelial and breast carcinoma cells.

In vivo activity of D-reverse peptides from the type 1 repeats.

In addition to demonstrating their activity in breast cancer xenografts, we have examined the activity of the D-reverse peptides in other animal disease models that involve physiological or pathological angiogenesis. Through a collaboration with Dr. Luisa Iruela-Arispe (Appendix F), we examined the activity of TSP1 type 1 repeat peptides in the chick chorioallantoic membrane (CAM) angiogenesis assay. This assay assesses anti-angiogenic activity with respect to developmental angiogenesis during embryonic development. Thus, the assay is generally regarded as a more pure assessment of anti-angiogenic activity than tumor

xenografts, where the peptides may affect both the tumor cells and endothelial cells. We demonstrated that both the native and D-reverse peptides are active in this assay (see Fig. 6 in Appendix F). The somewhat higher activity of the D-reverse peptides in this figure may reflect their enhanced stability. We demonstrated that the portion of this peptide that mediates activation of latent TGFβ was not required to inhibit angiogenesis in the CAM assay. To determine whether activity in the CAM assay was predictive of anti-tumor activity for breast cancer, we compared peptides with or lacking the TGFβ-activating sequence for inhibiting growth of MDA-MB-435 tumors in mice (Appendix F, Fig. 9). These data demonstrated that the TGFβ-activating sequence is not essential for the anti-tumor activity of these peptides.

Surprisingly, we found using this assay that the type1 repeat peptide we have studied preferentially inhibits FGF2 but not vascular endothelial growth factor (VEGF)-induced angiogenesis (Appendix F, Fig. 8). In contrast, a second peptide from this region that binds to CD36 inhibited both FGF2 and VEGF-stimulated angiogenesis in the CAM assay. This suggest that these two peptides act through distinct signaling pathways to suppress angiogenic responses and demonstrate that two sequences from the type I repeats of TSP1 can independently inhibit angiogenesis in a physiological model.

The D-reverse peptides were also tested for activity to inhibit angiogenesis in animal models of glomerulonephritis (33) and brain cancer (34). Although these collaborative projects were not supported by the Army funding, they provided further insights into the activity of these peptides and demonstrate that the anti-tumor activity of these peptides is not limited to breast cancer. As part of both studies, we examined the use of Alzet pumps to continuously deliver the peptides. Although this route of delivery produced activity, it was not superior to daily intravenous administration. The brain tumor model also provided an opportunity to begin to define the pharmacokinetics of the peptides (34). We demonstrated that the D-reverse peptides have biphasic clearance from circulation with t1/2=0.056 h, r^2 =0.998 via kidney excretion and a slower component with t1/2=50.5 h, r^2 =0.98. Fluorescently labeled peptide was demonstrated to preferentially accumulate in the tumor vasculature compared to that observed in normal vascular beds. This suggests that the peptides have potential as targeting ligands for the tumor vasculature.

Structural requirements for α3β1 mediated adhesive activity of TSP1

Based on the major role we discovered for the α3β1 integrin in mediating TSP1 interactions with both breast carcinoma ((35), Appendix D) and endothelial cells (manuscript submitted, Appendix G), we also defined peptide sequences from TSP1 that mediate this interaction (Appendix E). This work was supported both by intramural NIH funds and the Army grant. We localized the region of TSP1 recognized by the α3β1 integrin using recombinant fragments and synthetic peptides to amino acids residues 190-201 of TSP1, which promoted adhesion of MDA-MB-435 breast carcinoma cells when immobilized and inhibited adhesion of the same cells to TSP1 when added in solution. Adhesion to this peptide was enhanced by a β1 integrin activating antibody, Mn^{2+}, and insulin-like growth factor I (IGF1) and was inhibited by an α3β1 integrin function-blocking antibody. The soluble peptide inhibited adhesion of cells to the immobilized TSP1 peptide or spreading on intact TSP1, but at the same concentrations did not inhibit attachment or spreading on type IV collagen or fibronectin. Substitution of several residues in the TSP1 peptide with Ala residues

abolished or diminished the inhibitory activity of the peptide in solution, but only substitution of Arg(198) completely inactivated the adhesive activity of the immobilized peptide. The essential residues for activity of the peptide as a soluble inhibitor are Asn(196), Val(197), and Arg(198), but flanking residues enhance the inhibitory activity of this core sequence, either by altering the conformation of the active sequence or by interacting with the integrin. This functional sequence is conserved in all known mammalian TSP1 sequences and in TSP1 from *Xenopus laevis*. The TSP1 peptide also inhibited adhesion of MDA-MB-435 cells to the laminin-1 peptide GD6, which contains a potential integrin-recognition sequence Asn-Leu-Arg and is derived from a similar position in a pentraxin module. Adhesion studies using recombinant TSP1 fragments also localized β1 integrin-dependent adhesion to residues 175-242 of this region, which contain the active sequence.

Recently, we have prepared a D-reverse analog of the α3β1 integrin binding sequence and demonstrated its activity to inhibit adhesion to TSP1 *in vitro* and to inhibit angiogenesis in the chick chorioallantoic membrane angiogenesis assay. Thus, the strategy we used successfully to develop stable analogs of the type 1 repeat peptides can be applied to develop angiogenesis inhibitors based on this novel TSP1 peptide. These novel peptide analogs may have unique activities as therapeutic angiogenesis inhibitors and may act synergistically with the type 1 repeat peptide analogs. These questions will be explored in our future efforts.

TASK 2: Preparation of polymeric conjugates.

METHODS

Preparation of polysucrose conjugates-- Polysucrose of average molecular weight of 70,000 or 400,000 (Ficoll, Pharmacia) was first functionalized with primary amino groups as previously described (36). This derivative, referred to as AECM-Ficoll (50 mg) was iodoacetylated in 1.35 ml of 0.15 M HEPES-NaOH buffer at pH 7.5 containing 1 mM EDTA by addition of 9.6 mg of iodoacetic acid N-hydroxysuccinimide ester (Sigma) dissolved in 0.15 ml of dimethylformamide. After about 15 min of reaction, the solution was passed over a desalting column to obtain the iodoacetylated AECM-Ficoll. Nine micromoles of peptide were dissolved in 1.8 ml of distilled water, and 250 µl of a 50 mM solution of tris-(2-carboxyethyl) phosphine hydrochloride (Pierce Chemical) in water was added to the peptide solution, and the pH was adjusted to 7.1 to 7.8 by addition of 1 M Na_2CO_3. After 30-60 min, the resulting solution was passed through a column packed with 1.4 ml of BioRad AG1-X8 anion exchange resin in the acetate form. The column effluent was led into the iodoacetylated AECM-Ficoll solution and the solution was stirred overnight at room temperature. The resulting solution was then dialyzed overnight against phosphate-buffered saline with several changes in a 12-14 kDa molecular weight cutoff tubing. Peptide concentration of the resulting solution was determined by measuring its absorbance at 280 nm using E = 5540 $M^{-1}cm^{-1}$ per Trp residue.

RESULTS

The peptide conjugates were prepared and demonstrated enhanced activity when tested *in vitro* for inhibiting heparin binding to TSP1 or for inhibiting proliferation of endothelial cells stimulated by FGF2 (Appendix B, Fig. 4 and Table 3). However, data published in

Appendix B, Fig. 5, demonstrated that the polymer conjugates did not have significant activity in the breast tumor xenograft model. Therefore, we have not pursued a thorough optimization of these conjugates as described in the Statement of Work. In studies with a collaborator, we have found that the polymeric conjugates have biological activity when used to treat retinopathy of prematurity in a rat model (Shafiee et al, manuscript submitted, Appendix ?). Thus, these conjugates do exhibit anti-angiogenic activity *in vivo* and may be useful as therapeutics for diseases other than breast cancer. The lack of activity of the conjugates in breast cancer may be due to a decreased bioavailability of the peptides in breast tumors when conjugated to polysucrose.

TASK 3: Preparation of site-directed mutants of thrombospondin-1

For specific aims 2 and 3, we have completed transfections with four full length *THBS1* cDNA expression vectors containing point mutations in the type I repeats. Stable transfected cell lines expressing high levels of two of these constructs have been prepared, but stable lines expressing the other two constructs could not be obtained (W385A and F432A). In this final report, we present further characterization of the *in vitro* interactions of mutant and wild type TSP1 with breast carcinoma and endothelial cells.

a) Construction of mutant expression vectors and verification of sequence:

METHODS

We have prepared expression constructs containing four directed mutations of type I repeat sequences in TSP1 as follows.

The full length expression vector pCMVTHBS1 was used for preparation of site-directed mutations. Mutations in the central Trp residues of each Type I repeat and TGF beta activation sequence in the second repeat were prepared by filling a gapped plasmid in the presence of mutant primers using the linker scanning method (37). This method introduces a mutation directly into the double stranded plasmid template. Briefly, a frame of single stranded DNA encompassing only the region of interest is created within a double stranded plasmid (Fig. 1). The mutagenic oligonucleotide is used as a primer for the Klenow fragment of DNA Polymerase I that synthesizes the second strand of the target region and ligation is done using T4 DNA ligase. The schematic representations of the methods used are shown in the figure. The ligation mixtures were transformed into a *mutS* strain of E. coli, BMH 17- 81, with a defect in mismatch repair.

Screening for the clones that had the mutation was first done using 'Touchdown' PCR. This method minimizes mispriming of a specific primer that is designed to be an exact match at the 3' end to the mutant sequence by raising the temperature of primer annealing in the initial cycles of PCR. Thus, mutant products are preferentially amplified in the initial cycles and can preferentially serve as the template for amplification in the following cycles. The primers used for Touchdown PCR are listed below.

THBS W385A - Primers span bases 1025 to 1264 yielding a fragment size of 239 bp.

Forward primer : 5'- ATGAGCTGAGGCGGCC- 3'

Reverse Touchdown primer : 5'- AGGTCCACTCGGACGC- 3'

THBS W441A -Primers span bases 1250 to 1432 yielding a fragment size of 182 bp.

Forward primer : 5'- GGTCCGAGTGGACCTCCTG- 3'

Reverse Touchdown primer : 5'- ATGACCACGGGGACGC- 3'

THBS W498G - Primers span bases 1349 to 1605 yielding a fragment of 256 bp.

Forward primer : 5'- TCCAGACACGGACCTGC- 3'

Reverse Touchdown primer : 5'- GATGTCCCATGGTGACCC- 3'

THBS F432A - Primers span bases 1250 to 1405 yielding a fragment size of 155 bp.

Forward primer is the forward primer used to make THBS W441A

Reverse Touchdown primer : 5'- CACCATCCTGTTTAGC- 3'

Cell lysates from individual colonies obtained from the linker scanning mutagenesis were prepared by boiling in 40 µl of water for 5 minutes. 2 µl of the lysates were used as template for Touchdown PCR.

Plasmids from the selected clones were transformed into competent DH5alpha cells and validated by restriction analysis and complete sequencing of the DNA regions filled using the Klenow fragment. The remainder of the *THBS* coding sequence in each clone was screened for mutations by single strand conformation polymorphism (SSCP)-PCR using overlapping primer sets.

RESULTS

In order to study the role of potential anti-angiogenic sequences in the Type I repeats of TSP1 in regulating angiogenesis of breast and some other tumors, we performed site directed mutagenesis of an expression vector containing a full length THBS cDNA. Initial mutations were constructed to replace four amino acid residues shown to be critical for biological activities of synthetic peptides derived from the type I repeats. Central Trp residues in each type I repeat and the Phe residues required for activation of latent TGFβ were mutated to yield the following mutant thrombospondins: Trp(385)Ala, Trp(441)Ala, Trp(498)Gly, and Phe(432)Ala.

Touchdown PCR of mutant PCR fragments of thrombospondin using the respective specific primers showed distinct differences in the pattern of amplified products compared to native THBS sequence amplified using the same set of primers (Fig. 2). Lanes 2, 4 and 6 show the correct size product being amplified for the mutant PCR fragments. Comparing these to lanes 3, 5 and 7 respectively, either only the mutant product is amplified (lane 4 versus lane 5) or there is a difference in the patterns of mutant versus wild type (e.g. lanes 2 and 3). Once we established that we could differentiate mutant sequences using Touchdown PCR, we screened the bacterial colonies from the linker scanning method of mutagenesis. Figs. 3 and 4 show agarose gel electrophoresis of Touchdown PCR products from bacterial colonies screened for mutant sequences THBS W441A and THBS F432A. The positive clones were then sequenced between the fill-in sites to make sure other errors were not introduced by the enzyme used in the reaction. The mutant clone THBS W441A has a G > T substitution which changed a. a. 58 from Ala > Ser, and all other plasmids encoded the native TSP1 sequence except for the desired mutations.

WG1a is a mutant of wild type THBS clone that we obtained while preparing the mutant W498G DNA construct by the linker scanning method. Due to a two base pair deletion C at #1629 and A at # 1630), a frame shift and a premature stop codon were introduced at amino acid 516. Therefore, this protein lacks the carboxy terminus of TSP1 beyond the three type I repeats.

One other construct that we made was similar to the WG1a mutant. The N-terminus of THBS cDNA up to the end of third type I repeat (a.a.538) was cloned into an expression vector pCAGGS driven by a chicken beta actin promoter. This truncated mutant will be used to study the function of the type I repeats of TSP1 without interference from the other functional domains of TSP1.

b) Transfection and isolation of stable transfectants.

METHODS

MDA435 cells were transfected by electroporation using 10 μg of pCMVTHBS1 vectors containing the mutations listed in Table I (Appendix K) or pCMVneo vector control. Transfected cells were initially grown as a pool in complete medium. After 48 h, cells were selected as pools by growth in 700 μg/ml G418. After 2-3 weeks, resistant cells were cloned by seeding at limiting dilution in 96 well microtiter plates in medium supplemented with filtered conditioned medium from parental MDA435 cells. When the cells were subconfluent, the medium was replaced with 0.2 ml of serum free medium (CHO-S-SFM, Gibco BRL) containing G418. After 16 h, the conditioned medium was removed and stored at -70° for ELISA analysis. Colonies arising from single cells that secreted TSP1 were expanded and cryopreserved in liquid nitrogen.

The serum-free conditioned media were assayed for expression of TSP1 by a sandwich ELISA. Microtiter plate wells were coated with 5 ng of Heparin-BSA (Sigma) in 50 μl of PBS by incubating overnight at 4°. A 50 μl sample of each conditioned medium was added to the wells in 3-fold serial dilutions and incubated for 2 h at 37°. The stably transfected clone containing the full length wild type THBS1 sequence (TH26 or 29) was used as the positive control and a pCMVneo transfectant was used as a negative control. The wells were blocked by incubation in Tris-BSA. The wells were aspirated and incubated with 50 μl of 1:500 dilution of rabbit anti-TSP1 in Tris-BSA for 2 h at 37 ^{0}C. The wells were aspirated and washed 3 times with DPBS, 0.02% BSA, 0.02 mM phenylmethyl sulfonyl fluoride, 0.05% Tween 20 (DPBS-TWEEN). A 1:1000 dilution of peroxidase conjugated goat anti-rabbit IgG (Kirkegaard and Perry) was added and incubated for 1 h at room temperature. The wells were aspirated and washed 3 times with DPBS-TWEEN. o-Phenylenediamine substrate (Sigma P8412) was diluted in phosphate/citrate/perborate buffer (Sigma P4922), and 50 μl was added to each well and incubated for 7-10 minutes. Development was stopped by addition of 100 μl of 3 M sulfuric acid. The clones that were positive for expression of TSP1 were cryopreserved in liquid nitrogen.

Clones identified by this assay were re-screened by Western blotting of serum-free conditioned medium to verify the size of the recombinant TSP1 secreted by the cells. TSP1 on the blots was detected using rabbit antibody to denatured TSP1 and peroxidase conjugated

goat anti-rabbit IgG followed by visualization using ECL reagent (Amersham). Clones isolated by the above mentioned procedure were analyzed by RT-PCR to confirm they had the plasmid derived TSP1 mRNA.

RT-PCR analysis of clones expressing the mutant TSP1: Total RNA was extracted from transfected MDA cells. 4 µg of total RNA was used for the reverse transcription using M-MLV reverse transcriptase, and 10% of the RT reaction mixture was used as template for the PCR. In the first step of RT-PCR, an antisense primer was annealed to the mRNA at the 5' end of the thrombospondin sequence which would help transcription of the sequence upstream of the primer. The single stranded cDNA was then subjected to PCR using a nested antisense primer and a sense primer from the rabbit beta globin gene which is immediately upstream of the *THBS* gene in our expression vector. The rationale for doing this RT-PCR was to amplify only the transcript that was derived from the expression vector and not the endogenous *THBS* gene.

RESULTS

Stable transfectants were screened for over expression of mutant TSPs using a sandwich immunoassay with heparin-BSA as a capture ligand and rabbit anti-TSP1 as detecting antibody (Fig. 5). Using peroxidase conjugated secondary antibody and o-phenylenediamine for development, conditioned serum free media from 20 clones were screened in one day. Clones with high expression identified by this assay were re-screened by Western blotting to verify the size of the recombinant TSP1 secreted by the cells using a new antibody raised to reduced and alkylated TSP1 (Fig. 6). In previous transfections using wild type pCMVTHBS1, a single clone was obtained that had undergone rearrangement resulting in expression of a truncated TSP1 (47). In the present screenings we identified several additional clones with similar rearrangements (e.g. Fig 6, clone A.A11). Several of these were saved for examining the activity of type I repeat mutants in the context of C-terminal deletions, which we have previously shown to abrogate the anti-tumor activity of TSP1 over expression. Selected clones expressing full length TSP1 by Western analysis were analyzed by Northern blotting to verify that the increased expression is due to expression of the transgene rather than activation of the endogenous *THBS1* gene.

Three stable clones, AA11 expressing the truncated mutant, AE9 and EA3 over expressing the W441A mutant and clones A3D, C8E and C6E over expressing the W498G mutant have been isolated. However we were unable to make stable clones expressing theW385A and F432A mutant proteins. Although initial screening yielded several clones that expressed TSP1 of the correct molecular weight as evidenced by Western blot analysis, further examination of the origin of the TSP1 expressed in these clones by RT-PCR showed that they were negative for the mutant THBS mRNA. The well characterized serum response of the *THBS1* promoter was used to differentiate expression of the transfected mutant from expression of the normally silent endogenous *THBS1* gene (38). The serum induction of expression observed in these experiments indicated that the THBS F432A and W385A clones had up-regulated their endogenous *THBS1* gene and were not expressing the stably integrated mutant TSP1.

Construction of epitope-tagged THBS Expression vector: Although TSP-positive clones were obtained from the F432A transfectants, the above data indicates that these clones have induced their endogenous gene. Because of the difficulty in differentiating mutant TSPs from TSP1 produced by up-regulation of the normally inactive endogenous gene, we prepared an epitope tagged THBS expression plasmid. We have made a THBS expression construct which has the 9E10 epitope sequence (EQKLISEEDL) derived from the human c-myc protein followed by six histidine residues at the 3' end of THBS sequence. The vector was constructed as follows. The 3' end ClaI - BclI fragment of THBS was PCR amplified using a sense primer corresponding to the sequence from base 2935 to 2953 spanning the ClaI site and an antisense primer which contained the sequence for the myc-his tag and the 3' end cloning site BamHI incorporated into it. The product of this PCR reaction, a 779 bp fragment, was cloned into pCMV neoBam Vector along with two 5' end restriction digestion fragments of THBS, a 1.3 Kb BamHI-EcoRI fragment and a 1.6 Kb EcoRI- ClaI fragment. A clone which had the coding sequence of THBS with the myc-his tag in the correct orientation has been isolated.

This construct was transiently expressed by electroporation of MDA435 cells, and expression of the tagged thrombospondin was detected by metabolic labeling and immunoprecipitation using anti-myc antibody (Fig. 7). The myc-tagged thrombospondin can be detected in lysates of pulse labeled cells (lane 2) and is detected in the medium after chasing for 3 hours (lane 1). We will next introduce the mutations into the myc-his construct, and use the plasmids to distinguish between the endogenous and exogenous gene expression.

c) Purification of mutant proteins and characterization of binding activities and effects on endothelial cell proliferation.

METHODS

MDA cells transfected with mutant thrombospondin gene were grown in RPMI medium containing 700 µg/ml G418. When the cells were approximately 80% confluent, the medium was replaced with CHO-S-SFM II medium (Life Technologies) containing 700 µg/ml G418. The medium was collected 48 hours later and centrifuged in polypropylene tubes in a Sorvall RC-5B centrifuge using SS-34 rotor at 15,000 x rpm for 15 min. The supernatant from this step was passed thorough a 0.45 µm low protein-binding syringe filter. The filtered medium was immediately used for the purification of thrombospondin.

Heparin affinity chromatography: A 1 ml HiTrap Heparin column (Pharmacia Biotech) was used. The column was washed with starting buffer (10 mM Tris, pH 7.5 containing 150 mM NaCl, 1 mM $CaCl_2$, 0.1 mM phenylmethyl sulfonyl fluoride, 5 mM benzamidine and 1 mM N-ethylmaleimide). About 200 ml of the conditioned medium was passed through the HiTrap Heparin column at a flow rate of 1 ml/min. The unbound material was collected and discarded. The column was then washed with 15 ml of the above buffer followed by a 6 ml of the starting buffer containing 0.35 M NaCl. The eluted materials at this step were discarded. The column was then eluted with the starting buffer containing 0.6 M NaCl and the eluted protein fractions were collected and stored at -70° C. Partially purified

thrombospondin thus obtained from several batches were combined and concentrated by passing through a HiTrap heparin column.

Western blotting: Heparin affinity chromatography-purified TSP1 was electrophoresed by SDS-PAGE under reducing conditions as described above. The separated proteins were transferred to PVDF membrane by electro blotting and the membrane was then blocked with non-fat dry milk. The membrane-bound proteins were then probed with polyclonal antibody raised against platelet TSP1. Horse radish peroxidase-coupled secondary antibody and ECL reagent (Amersham) were used to visualize the antibody reactive bands.

Identification of complex formation between TSP1 and band-X : Two independent methods were employed to identify the interaction between TSP1 and band-X. In the first method, TSP1/band-X complex purified by heparin affinity column was incubated with anti-TSP1 antibody-coupled agarose beads. The incubation was carried out for 3 hours at 4° C and the gel was rinsed thoroughly with buffer to remove unbound materials. The agarose gel was then boiled in SDS-PAGE electrophoresis sample buffer containing β-mercaptoethanol, centrifuged and the supernatant was analyzed by SDS-PAGE.

To examine the molecular size of band-X and its subunits, agarose gel electrophoresis was carried out under reducing and non-reducing conditions. 3% agarose gels were prepared in 0.5 M Tris buffer pH 8.8 containing 0.1% SDS. TH26 TSP1 containing band-X purified from heparin affinity chromatography was iodinated using iodogen (Pierce Chemical Co. IL) based on the manufacturer's protocol. The ^{125}I-labeled sample was then boiled in electrophoresis sample buffer with or without β-mercaptoethanol. Samples were then loaded onto agarose gel and electrophoresis was carried out at 100 volts for 1 hour. The gel was then dried onto a filter paper and exposed to film.

In the second method, TSP1 purified from human platelets was used to examine its interaction with band-X protein. ^{125}I-radiolabeled TSP1 was used as the binding ligand in this experiment. The binding was carried out in 96-well ELISA plates using a band-X preparation from heparin affinity chromatography column. Some of the 0.65 M NaCl-eluted fractions contained exclusively band-X, which typically eluted at the trailing end of the protein peak. These fractions were pooled, concentrated and coated onto the wells of an ELISA plate. The coating was done at 4° C overnight using different dilutions of band-X. The wells were then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). BSA-coated wells were used as control. ^{125}I-labeled TSP1 (150,000 cpm/per well) was then added and the plate was incubated at room temperature for 2 hours. The contents of the wells were then removed and the wells were gently rinsed with PBS. Individual wells were then removed from the plate using a pair of scissors and placed in counting vials. The radioactivity was measured using a gamma counter (Packard).

Characterization of the band-X protein was done by mass spectrometric analysis. The first step was to purify the TSP1/band-X complex from the culture supernatant by loading onto a Heparin-Agarose column and eluting the complex using 20 mM Tris buffer, pH 7.4, containing 0.65 M NaCl and 0.1 mM $CaCl_2$. The fractions containing the TSP1/band-X complex were reduced and acetylated before loading on the Sephacryl 400 gel filtration column. TSP1 and band-X were eluted out of the gel filtration column using 20 mM Tris-HCl

buffer, pH 7.6 containing 4 M Guanidine, 150 mM NaCl and 0.1 mM $CaCl_2$. The appropriate fractions containing the TSP1 and band-X were pooled, dialyzed extensively against water containing 0.02% Tween 20 and finally against water containing 0.02% Tween 20 and 1M ammonium bicarbonate. The dialyzed samples were lyophilized and redissolved in a smaller volume of water before analyzing them using 5% PAGE containing 6M Urea. The band corresponding to band-X was cut out of the Coomassie R 250 stained gel and analyzed by mass spectrometry.

RESULTS

While purifying the recombinant thrombospondins from the stable MDA435 cell lines, we discovered that the thrombospondin was tightly complexed with another secreted protein. This protein is referred to as band-X.

All purified TSP1 preparations except TH50, a C-terminus truncated form of thrombospondin, were found to contain band-X, (Fig. 8). Of the different cell lines tested, TH26-TSP1 was found to contain high levels of band-X secreted into the culture medium (Fig. 8; lane 4). During the purification TH26-TSP1, the band-X protein co-eluted with TSP1 from the heparin affinity chromatography column (Fig. 9; lane 2). Several methods were tested for the separation of these two proteins including gel filtration, ion-exchange chromatography under denaturing and nondenaturing conditions, lectin affinity chromatography and barium citrate precipitation method as described by Alexander et al. (39). However, thrombospondin and band-X protein consistently co-purified in each of these chromatographic methods.

Properties of band-X protein : The molecular weight of band-X protein was estimated to be 230-240,000 based on its electrophoretic mobility in SDS-PAGE. Band-X was readily digestible with trypsin or thrombin, showing that it is a protein molecule (Fig. 10). The composite results from this experiment and the lectin binding properties of band-X suggest that it is a glycoprotein. Figure 11 shows the results of Western blotting studies using different TSP1 preparations probed with anti-TSP1 polyclonal antibody. Although this antibody recognized all of the recombinant TSP1 preparations, it did not interact with band-X protein. In a separate experiment, this antibody was coupled to agarose gel and this immunoaffinity gel was used to examine TSP1 interaction. When TSP1/band-X mixture was incubated with the immunoaffinity gel, the bound material upon analysis by SDS-PAGE was found to contain band-X. This result suggests the possibility of a stable complex formation between TSP1 and band-X, and therefore the two molecules co-precipitated together. Control agarose gels without the antibody did not bind TSP1 or band-X.

To further examine the interaction between TSP1 and band-X protein, ^{125}I-labeled TSP1 was allowed to interact with substratum-bound band-X protein in ELISA plates. Band-X protein was coated in decreasing dilution and no decrease in radiolabeled TSP1 binding was noticed up to a dilution of 1:8 (Fig. 12). However, further dilutions of band-X showed a dose dependent decrease in TSP1 interaction. These results indicate that TSP1, independent of its source, binds to band-X. When the radiolabeled proteins were resolved in a gel filtration column, the TH26-TSP1/band-X preparation was found to elute at the same point as platelet TSP1 (Fig. 13), showing the anomalous behavior of the TSP1/band-X complex. It is also

possible that the condition applied in the gel filtration column separated the complex into individual proteins, but due to their close molecular weight they both eluted as a single peak. Electrophoretic analysis of the eluted peak showed the presence of both proteins.

To study the electrophoretic mobility of band-X protein under non-reducing condition, agarose gel was used for electrophoresis. In this method, TH26-TSP1 and band-X together appeared as a single band under non-reducing condition suggesting that the two molecules have a similar molecular weight in their native form (Fig. 14; Lane 1). However, due to the difference in their subunit molecular weights, band-X and TH26-TSP1 appeared as two independent bands after reduction of disulfide bonds (Fig. 14; Lane 2). This result suggests that band-X protein may contain two subunits with similar molecular weights. The electrophoretic mobility of platelet TSP1 under reducing and non-reducing condition in agarose gel is shown in Fig. 14 lanes 3 and 4 for comparison.

The band that was tightly bound to the TSP1 protein was purified by preparative SDS gel electrophoresis, digested in gel with trypsin, and identified by mass spectrometry to be fibronectin secreted by MDA 435 cells. Since it is extremely difficult to separate the two proteins from each other in the conditioned media of stable MDA 435 clones, we decided not to pursue purification of mutant TSP1 proteins from the stably transfected MDA 435 clones. A protein of similar molecular weight also co-purified with TSP1 when over-expressed in a murine melanoma cell line that does not express endogenous murine TSP1 (K1735 clone TK) (40). Therefore this cell line could not be used to purify mutant thrombospondins. Preliminary microarray experiments performed in the lab has shown that Jurkat T lymphoma cells do not express endogenous fibronectin. Thus, Jurkat cells can be potentially used for the over expression of mutant thrombospondins. This work will be continued in the future to complete this task for publication.

TASK 4: Determine effects of expression of mutated thrombospondin-1 on behavior of breast carcinoma and endothelial cells.

a) Characterize level and stability of expression of mutant transfectants.

METHODS

The level of expression of mutant transfectant proteins was assayed by the sandwich ELISA and by Western blotting as mentioned earlier.

Metabolic Labeling and Immunoprecipitation of TSP1: 2x 10^6 MDA435 cells were transfected with 10 µg each of the control vector, wild type THBS expression construct or the mutant DNA constructs by electroporation in a total volume of 25 µl. The electroporated cells were plated into tissue culture plate and allowed to grow for 24 hours in complete growth medium. At the end of 24 hours, cells were washed twice with methionine-free, serum-free medium and incubated at 37^0 C for another 1 hour in the same medium. The medium was aspirated from the wells and incubation was continued with 1 ml of methionine-free medium supplemented with 100 µCi [^{35}S]-methionine. For pulse-chase experiments, after 30 minutes

of labeling, monolayers were washed with growth medium, re-fed with 2 ml of growth medium supplemented with 0.2 mM unlabeled methionine and incubated for 30, 60 or 180 min.

After the metabolic labeling, the spent media from the wells were collected. The monolayers were lysed in 0.3 ml of RIPA buffer (50 mM Tris, pH 7.4 containing 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylmethyl sulfonyl fluoride and 10 µg/ml of Protease inhibitor cocktail) on ice for 10 minutes, and the cell lysates were centrifuged to remove debris. 200 µl aliquots of conditioned media and 150 µl aliquots of the cell lysates were used for immunoprecipitation of TSP1 with 6 µl of polyclonal anti-TSP1 antibody at 4^0C for 1 hr. After 50 µl of Protein A- agarose was added, samples were further incubated at 4^0C overnight. The immune complex which precipitated with Protein A- agarose was washed extensively with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 1% NP-40. The final washed pellets were boiled in SDS- PAGE loading buffer followed by electrophoresis on a 4-15% gradient gel.

Transient Transfection Experiments: Transfection of MDA435 cells was routinely done by electroporation (Cell Porator, Life Technologies, Gaithersburg, Maryland) in precooled micro electroporation chambers with a 0.15 cm gap between the two flat-topped bosses. The chilled mixture of $2x10^6$ cells and 10 µg DNA in a total volume of 25 µl of growth medium (RPMI 1640 supplemented with 10% FBS) was placed between the electrode bosses and electroporations were performed with an electrical pulse from a 50 µF capacitor charged to 150 V. The chambers were placed on ice for 15 min. after electroporation and then plated into one well of a 6-well tissue culture plate containing 3 ml of growth medium.

RT-PCR Analysis of tumor tissue: Total RNA was extracted from frozen samples of mammary fat pad tumors from mice that were injected with transfected MDA cells. 4 µg of total RNA was used for the reverse transcription using M-MLV reverse transcriptase, and 10% of the RT reaction mixture was used as template for the PCR as mentioned above.

RESULTS

Stability of expression of mutant transfectants was determined three ways to allow us to verify expression of several mutated thrombospondins. However, failure to isolate stable transfectants expressing F432A TSP1 and W385A TSP1 could indicate instability of these mutant proteins or their mRNA or an inability of the cells to process or secrete these mutant proteins. Lack of secretion of erythropoietin receptor following mutation of its WSXW sequence homologous to the thrombospondin type I repeats (41, 42) is consistent with this hypothesis. Alternatively, the mutant protein may be expressed but could be toxic to the cells. In order to determine if the expression of the mutant F432A protein was toxic to the MDA435 cells, we conducted green fluorescent protein (GFP) co-transfection experiments. Briefly, 2x 10^6 cells were transfected with 10 µg of wild type THBS expression construct or F432A mutant construct along with 2.5 µg of pGreen Lantern -1 DNA (Life Technologies, Gaithersburg, MD) by electroporation (43). The electroporated cells were plated into tissue culture plates and allowed to grow for 24 or 48 hours. At the end of each time point, the cells

were viewed by fluorescent microscopy. Total number of cells and cells positive for GFP under fluorescent light were counted in three different fields of view for each of the transfections. The results of this experiment are presented in Table 2. At 24 hours, there was over 50% reduction in the number of GFP-positive cells in the F432A transfected cells compared to wild type THBS transfected cells; at 48 hours, only 30% of the mutant transfectants were GFP-positive compared to wild type THBS transfectants. Therefore, expression of the F432A mutant may cause cell death or inhibit growth.

To examine the alternate hypothesis that expression of the mutant proteins in MDA435 cells was difficult to achieve because the mRNA for that transgene was very unstable or the mutant protein failed to be secreted, we used pulse labeling to compare synthesis of the mutant and wild type proteins under low serum conditions. In order to compare the stabilities of wild type versus the mutant TSPs, we transiently transfected MDA435 cells with different DNA constructs, pulse-labeled them with [^{35}S] methionine and chased with cold methionine for 30, 60 and 180 min. Cells transfected with pCMV control vector showed minimal synthesis and secretion of TSP1 after a 30 min. Pulse labeling and chase for 60 or 180 min with unlabeled methionine. Cells transfected with wild-type THBS vector showed high levels of TSP1 in the cell lysate after a 60 minute chase and secreted TSP1 was detected in the medium after 3 h (Fig. 15). Cells transfected with the W385A and F432A mutant THBS plasmids showed similar expression and secretion as the wild type transfectant, indicating that synthesis and secretion of these mutant thrombospondin proteins is normal. Similar results were obtained with the other mutant proteins.

Another method used to detect the stability of the mutant protein was to examine the tumors formed in the mammary fat pads of mice for expression of the plasmid to verify that expression of the mutated TSP1 was retained throughout tumor growth in the animals. In the case of the stable clones of W441A that were implanted in the mammary fat pads of nude mice, at sacrifice, portions of each tumor were frozen in liquid nitrogen for preparation of total RNA. RT-PCR was used to confirm the tumors had the plasmid-derived TSP1 mRNA. Figure 16 shows the products of RT-PCR of tumor RNA from animals which received *THBS* W441A clones. Every one of the tumors showed the expected product, although the amount of expression varied from tumor to tumor. The mean expression of the plasmid-derived TSP1 RNA in the tumors shown in lanes 3, 4 and 5 are lower than the mean from tumors shown in lanes 6, 7 and 8. Tumors in lanes 3, 4 and 5 and 6, 7 and 8 came from mice injected with clones AA11 and EA3 of *THBS* W441A respectively. This is interesting because while wild-type TSP1 expression in tumors has shown to produce smaller primary tumors and a reduction in capillary densities (47, 58) the tumors from the clone AA11 with a spontaneous truncation mutation similar to TH50 are bigger compared to the ones from other clones.

b) characterize behavior of transfected cell clones in vitro: To circumvent the problem we faced purifying mutant proteins from stably transfected clones, we followed two different strategies.

METHODS

Transient transfection and uptake of ^{3}H- thymidine: Bovine aortic endothelial cells

(BAE Cells) between passages 3 and 13 or MDA435 breast cancer cells between passages 4 and 20 were used for transient transfections by plasmid expression vectors carrying the wild type and type I repeat mutant cDNAs of TSP1. Transfections were done by electroporation. The protocol used for transfection is briefly outlined as follows.

MDA435 cells or BAE cells were trypsinized and resuspended in growth medium. $2x10^6$ cells were aliquoted into sterile microfuge tubes containing varying amounts of THBS DNA constructs used for transfection and 5 μg of a plasmid expression vector for β-galactosidase. The total volume was adjusted to 25 μl and incubated on ice for 20 minutes. The cell-DNA mixtures were then transferred into prechilled electroporation chambers taking care to suspend them between the two electrode bosses. The mixtures were exposed to a 1 sec. pulse from a 50 μF capacitor charged to 150 volts. Electroporation chambers were immediately placed on ice for another 20 min. The mixture was then resuspended into 3 ml of medium containing 1% FCS (for BAE cells) or 10% FCS (for MDA435) and plated into two wells of a Nunc 6 well plate. One well was used for ^{3}H-thymidine labeling and the other was used for protein and β-galactosidase activity assays.

24 hr after the transfection, the medium was replaced with 1 ml of medium supplemented with 1 or 10% FCS and one well from each transfection was labeled with 2.5 μCi of ^{3}H-thymidine/ml medium. Labeling was continued for 4 hr at 37^0 C in a CO_2 incubator. At the end of incubation the cells were washed twice with DPBS, fixed with methanol/acetic acid and extracted with 600 μl of trypsin/versene at 37^0 C for 1 hr and at RT for 30 min. 200 μl of 1% SDS were added to the cell extracts which were then counted using a scintillation counter to determine the total uptake of ^{3}H-thymidine.

The well containing the other half of the transfected cells was washed and extracted with 300 μl of ice cold extraction buffer (0.1 M sodium phosphate, pH 7.4 containing 0.5% triton X-100). The cell extracts were freeze-thawed three times and cell lysates were cleared by centrifugation at 12,000g for 15 min at 4^0 C. Aliquots of cell lysates were assayed for total protein content by the BCA method and for β-galactosidase activity using nitrophenyl β-galactopyranoside as substrate. The β-galactosidase activities of the cell lysates were expressed as mU/μg protein standardized using purified *E coli* β-galactosidase (Sigma).

Bioassay for inhibition of endothelial and breast carcinoma cell proliferation: Proliferation of bovine aortic endothelial cells was determined as previously described (30) in the presence of recombinant fragments of TSP1 as GST fusion proteins.

RESULTS

MDA435 cells were transiently transfected with varying amounts of *THBS1* wild type (WT) DNA or mutant DNA construct to study their effects on the proliferation of the cells. Proliferation was measured as the total uptake of ^{3}H- thymidine by transiently transfected MDA435 cells. As shown in Figure 17 A, when the assay was done in the presence of 10% FCS, dose-dependent inhibition of proliferation was seen which was maximal at a DNA concentration of 24 μg in the case of cells transfected with THBS-WT DNA. Under similar conditions F432A DNA transfected cells showed very little inhibition of proliferation, even though the transfection efficiency remained above 100% of that for the β-galactosidase indicator vector alone (Fig. 17 B). Inhibition of thymidine incorporation in this assay is a

measure of DNA synthesis in the bulk population, the majority of which are not transfected, as assessed by X-Gal staining using the β-galactosidase reporter. Thus inhibition following transfection using wild type THBS vector results from accumulation of TSP1 in the medium which is secreted by the minority of transfected cells. Failure of the F432A mutant to inhibit thymidine incorporation indicates that this protein does not accumulate. This probably results from the rapid loss of cells expressing this mutant as detected by the GFP reporter studies.

Although we have demonstrated that platelet thrombospondin-1 inhibits proliferation of MDA435 cells (26), endothelial cells are much more sensitive to thrombospondin and are believed to be the physiological target for its anti-angiogenic activities. Effects of transient expression of wild type TSP1, the KRFK mutant F432A and the type I repeat mutant thrombospondins on bovine aortic endothelial cells were assayed in a similar manner, and the results are presented in Table 3. With increasing amounts of wild type *THBS* DNA used for transfection, a dose dependent inhibition of BAE cell proliferation is seen, while the transfection efficiency remains above control levels using the β-galactosidase vector alone. We also transfected BAE cells with 15-20 μg of the mutant DNAs. In the case of BAE cells transfected with W385A mutant construct, 15 μg of the DNA used for transfection did not show inhibition of incorporation of ^{3}H- thymidine. On the contrary, comparable amounts of W441A and W498G mutants had inhibitory effects similar to that of 18 μg of WT DNA.

WG1a is a mutant of wild type THBS clone that we obtained while preparing the mutant W498G DNA construct by the linker scanning method. Due to a two base pair deletion © at #1629 and A at # 1630), a frame shift and a premature stop codon were introduced at amino acid 516. Therefore, this protein lacks the carboxy terminus of TSP1 beyond the three type I repeats. Transient transfection of BAE cells with 16.5 μg of the WG1a mutant strongly inhibited BAE cell proliferation, with only 20% ^{3}H-thymidine being incorporated into the cells compared to the control. Transfection efficiencies in all the cases were high (Table 3), indicating that expression of this construct was not toxic to the cells.

Alternatively we used bacterial fusion proteins to map the antiproliferative activities of thrombospondin. Proliferation of bovine aortic endothelial cells was determined in the presence of recombinant GST-fusion proteins expressing the Type I, Type II, Type III and C-terminal domains of thrombospondin (Fig. 18). Procollagen and Type I repeat domains showed a dose-dependant inhibition of endothelial cell proliferation compared to medium control or GST control. At a concentration of 25 μg/ml, the recombinant procollagen domain inhibited BAE cell proliferation by about 35%, and at the same concentration the Type I repeat showed an inhibition of over 80% of control. The other recombinant fragments tested in this assay did not show a dose-dependant inhibition of BAE cell proliferation. These data demonstrate a specific antiproliferative activity for endothelial cells in the type I repeats and demonstrate a differential role of β1 and β3 integrins in adhesion of the two cell types. The RGD sequence in the type III repeats is recognized by endothelial cells but not by the breast carcinoma cells, which accounts for differential recognition of TSP1 fragments by these two cell types.

We have now identified the α3β1 integrin as the primary receptor on two breast carcinoma cell lines for TSP1. A published manuscript describing this work is attached (Appendix D) and is summarized below.

The α3β1 integrin, with some cooperation of sulfated glycoconjugates and α4β1

integrin, mediates adhesion of MDA-MB-435 and MDA-MB-231 breast carcinoma cells to TSP1. This β1 integrin is maintained in an inactive or partially active state in these cell lines but can be activated by exogenous stimuli including serum, insulin, IGF1 and ligation of CD98. In MDA-MB-231 cells, the inactive state of the α3β1 integrin is maintained by a G-protein mediated signal, but this suppression can also be overcome by IGF1 receptor signaling. Stimuli that increase β1-dependent adhesion to TSP1 do not stimulate β3-dependent adhesion to TSP1, even though the cells express the known TSP1 receptor αvβ3 and this integrin is functional and inducible for vitronectin adhesion. We do not know why the αvβ3 integrin on MDA-MB-435 cells can not recognize the RGD sequence in the type III repeat of platelet TSP1. Other cell types, however, can utilize the same TSP1 preparations used for these experiments to support αvβ3-dependent adhesion (44).

The α3β1 integrin in MDA-MB-435 cells does not recognize the RGD sequence in the TSP1 type 3 repeats. Because we have tested 85% of the TSP1 primary sequence using recombinant fragments or synthetic peptides, the β1 recognition motif may not be a linear epitope in TSP1. One caveat in interpreting the negative results using the recombinant TSP1 fragments, however, is that misfolding of fragments expressed in bacteria could selectively mask a linear recognition sequence for the α3β1 integrin in the GST- or T7-fusion proteins . Several β1 integrins have been implicated as TSP1 receptors in other cell types, including α2β1 on activated platelets (45), α3β1 on neurons (46), and α4β1 and α5β1 on activated T lymphocytes (47). α3β1 is the dominant integrin for mediating adhesive activity of breast carcinoma cells for TSP1, whereas α2β1 mediates adhesion of these cells to type I collagen but not to TSP1. The integrin α4β1 may play some role in breast carcinoma adhesion to TSP1, as we previously reported for T lymphocytes (47). The mechanism for the apparent differential recognition of TSP1 by β1 integrins among these cell types remains to be defined. However, it is notable that even within the breast carcinoma cell lines, pharmacological and physiological stimuli can differentially modulate activity of the α3β1 integrin for promoting adhesion or chemotaxis to TSP1. This finding implies a complex signaling process that regulates the recognition of pro-adhesive signals from TSP1 in the extracellular matrix. Both the IGF1 receptor and CD98 are components of this regulatory complex in breast carcinoma cells, but the mechanisms of their actions also remain to be defined.

Although several signaling pathways have been identified that regulate integrin activity by "inside-out" signaling (48), the mechanisms for regulating activation states of specific integrins remain poorly understood. In contrast to αvβ3 integrin, the α3β1 integrin in breast carcinoma cells is not activated by engagement of CD47 by the TSP1 "VVM" peptides or by protein kinase C activation. Rather, inhibition of Ser/Thr kinase activity, but not Tyr kinase activity, increases β1-mediated adhesive activity of MDA-MB-435 cells for TSP1. Conversely, phorbol ester activation of protein kinase C increased adhesion via αvβ3 but not α3β1 integrin. Thus, activation of individual integrins in MDA-MB-435 cells can be differentially regulated.

We have identified the IGF1 receptor as a specific regulator of α3β1-mediated interactions with TSP1. The insulin and IGF1 receptors were reported to be physically associated with the αvβ3 integrin but not with β1 integrins in fibroblasts (49). The αvβ3 integrin also co-immunoprecipitated with insulin receptor substrate-1 (50). Engagement of αvβ3 integrin by vitronectin but not α2β1 integrin by collagen increased mitogenic signaling

through the insulin receptor (49, 50). Thus, the specific activation of α3β1 mediated spreading and chemotaxis to TSP1 by insulin or IGF1 was unexpected. We observed a stronger response for stimulating adhesion to TSP1 than to collagen or laminin, suggesting that the regulation of avidity by the IGF1 receptor is specific for the α3β1 integrin. Other growth factors that utilize tyrosine kinase receptors including FGF2 and EGF did not activate this integrin. We therefore predict that specific coupling of α3β1 activation to IGF1 receptor signaling, rather than a general phosphorylation signal, mediates activation of the TSP1 binding integrin in breast carcinoma cells. The mechanism for this specific signaling remains to be determined.

We have also identified a TSP1 peptide that modulates angiogenesis and the in vitro behavior of endothelial cells through binding to α3β1 integrin. A submitted manuscript describing this work is attached and is summarized below (Appendix G).

We have demonstrated that sparse endothelial cells recognize an α3β1 integrin-binding sequence in TSP1 that stimulates endothelial cell spreading and proliferation when immobilized on a substratum. Addition of this peptide in solution inhibits endothelial cell spreading on TSP1, endothelial cell migration *in vitro* (Appendix G, Fig. 8), and angiogenesis *in vivo* (Appendix G, Fig. 9), presumably by inhibiting TSP1 interactions with this integrin. We have also demonstrated that the activity of this integrin to recognize TSP1 is suppressed in a confluent endothelial cell monolayer (Appendix G, Fig. 1). Loss of endothelial cell-cell contact during wound repair in vitro or angiogenesis *in vivo* could therefore activate this receptor and make the endothelial cells responsive to TSP1 signaling through the α3β1 integrin. Thus, recognition of immobilized TSP1 by α3β1 integrin may increase endothelial cell proliferation and motility during wound repair and angiogenesis *in vivo*.

c) Conduct animal studies of tumorigenesis and spontaneous metastasis.

METHODS

Tumorigenesis assay in nude mice: NIH Nu/Nu mice or NIH Beige XID mice, approximately eight weeks of age were injected with 10^5 MDA MB435 cells by the mammary fat pad route. Wild type MDA cells were used for peptide studies; stable transfectants expressing full length wild type or mutant TSP1 were used to examine the effects of site-directed mutations in TSP1 on tumorigenesis. Mice were anesthetized with 150-200 µl i.p. of a 1:80 dilution in PBS of a solution containing 25 g. tribromoethanol in 12.5 ml tertiary amyl alcohol. The mammary fat pad was cleaned with ethanol and a 10 mm incision was made directly above the site of injection. Using a 0.1 ml Hamilton syringe and 27 gauge ½" needle, 10 µl of cell suspension, 1 x 10^5 cells in HBSS for nu/nu mice or 4 x 10^5 for Beige mice, were injected into the fat pad. The incision was closed using 1-2 Autoclips (9 mm, Clay Adams). Autoclips were removed 7 days post-injection.

8-10 animals are injected for each condition, per experiment. Animals were ear punched after injection for subsequent identification. Beginning at day 25 and continuing every day until day 50, the experimental animals for peptide treatment were injected i.v. (tail vein) 100 µl of the free peptide or ficoll conjugates. Animals implanted with transfected MDA cell lines were not treated. Primary tumor size was determined twice weekly by length x width x height measurement, and the animals were observed daily for general health. When

the primary tumor of any animal exceeded 20 mm in any dimension, all of the animals were sacrificed. The presence of metastases was determined by gross autopsy and examination of H & E stained sections of step sections of the lungs and draining lymph nodes. The primary tumors were removed, stripped free of other tissues, and weighed. At any time during the experiment, animals suspected of being in distress were sacrificed and examined as above.

RESULTS

Three transfectant clones of MDA MB435 with high levels of TSP1 expression, AA11, AE9 and EA3 from THBS W441A transfection were selected for *in vivo* animal experiments. In the animal experiment, each group had 8 animals that received one clone of transfected cells. After 84 days, the animals were sacrificed and the tumors were removed. Figure 19 shows the growth curves for the different clones in athymic nude mice. Clones from THBS W441A transfection had the same or larger tumor masses compared to controls.

Histopathological analyses of these tumors showed that the tumors produced by the W441A clone producing the largest tumors, EA3, had relatively little necrotic area in the tumors compared to the control transfectants, although the former tumors were much larger. The lack of necrosis in these large tumors implies that angiogenesis in the W441A tumors is more efficient than in the control transfectants. Examination of the lung sections showed that 7 out of 8 (87%) of the W441A clone EA3 had lung metastases, whereas none of the control animals had detectable lung metastases.

We have also repeated this experiment using Beige XID mice, deficient in NK, T and B lymphocytes, to examine whether inhibition of tumor growth by over expression of thrombospondin and the lack of inhibition by W441A thrombospondin required interactions with components of mouse immune system other than mature T cells that are functional in athymic nude mice but not in the Beige mice. This was important to examine since we observed increased infiltration of mononuclear cells in tumors formed by THBS transfectants (10), and thrombospondin was recently reported to modulate activation of NK lymphocytes (51). Our data demonstrate, however, that the *THBS* transfected MDA435 cell lines retain their inhibition of tumor growth in Beige mice, which lack NK cells, relative to control transfectants (Fig. 20). Furthermore, the transfectants over expressing the THBS W441A mutant do not show a growth inhibition, and one clone tested produced larger tumors than the control transfectant tested (Fig. 20). This provides further support for our hypothesis that anti-angiogenic activity accounts for the observed growth inhibition in the mouse xenografts and that an intact Trp motif in the second type I repeat is required for this inhibitory activity.

Although one animal experiment was done wherein stable clones expressing the W498G mutant TSP1 were implanted in nude mice, the tumor take was very poor (only 20% of the animals in the positive control group formed tumors) making it difficult to draw any conclusions from it. That experiment will be repeated in the future.

KEY RESEARCH ACCOMPLISHMENTS

- Development of stable thrombospondin-1 peptide analogs and demonstration of their

anti-angiogenic activity and anti-tumor activity for breast carcinoma xenografts.

- Discovery that TSP1 and the TSP1 type 1 repeat peptides selectively induce apoptosis of endothelial cells.
- Mutagenesis of full length TSP1 expression vectors.
- Discovery that α3β1 integrin is the major TSP1 receptor mediating adhesion and chemotaxis of breast carcinoma cells.
- Discovery that the activity of α3β1 integrin to interact with TSP1 is regulated by insulin-like growth factor-1 and CD98.
- Demonstration that α3β1 integrin plays an important role in regulation by TSP1 of endothelial cell proliferation *in vitro* and angiogenesis *in vivo*.
- Identification of a the peptide sequence in TSP1 recognized by the α3β1 integrin and demonstration that this peptide is also a potent inhibitor of angiogenesis.

REPORTABLE OUTCOMES

Manuscripts

1. Roberts, D. D.: Regulation of tumor growth and metastasis by thrombospondin-1. *FASEB J.* 10:1183-1191, 1996.

2. Guo, N., Krutzsch, H.C., Inman, J.K., and Roberts, D.D.: Thrombospondin-1 and type I repeat peptides of thrombospondin-1 specifically induce apoptosis of endothelial cells. *Cancer Res.* 57:1735-1742, 1997.

3. Guo, N., Krutzsch, H.C., Inman, J.K., and Roberts, D.D.: Anti-proliferative and anti-tumor activities of D-reverse peptide mimetics derived from the second type-I repeat of thrombospondin-1. *J. Peptide Res.* 50:210-221, 1997.

4. Chandrasekaran, S., Guo, N., Rodrigues, R. G., Kaiser, J., and Roberts, D. D.: Pro-adhesive and chemotactic activities of thrombospondin-1 for breast carcinoma cells are mediated by α3β1 integrin and regulated by insulin-like growth factor and CD98. *J. Biol. Chem.* 274:11408-11416, 1999

5. Iruela-Arispe, M. L., Lombardo, M., Krutzsch, H. C., Lawler, J., and Roberts, D. D.: Inhibition of angiogenesis by thrombospondin-1 is mediated by two independent regions of the type 1 repeats. *Circulation* 100: 1423-1431, 1999.

6. Krutzsch, H. C., Choe, B., Sipes, J. M., Guo, N., and Roberts, D. D.: Identification of an α3β1 integrin recognition sequence in thrombospondin-1. *J. Biol. Chem.* 274: 24080-24086, 1999.

7. Shafiee, A., Penn, J. K., Krutzsch, H. C., Inman, J. K., Roberts, D. D., and Blake, D. A.: Peptides derived from the type 1 repeats of thrombospondin-1 inhibit angiogenesis

in a bovine retinal explant assay and a rat model of retinopathy of prematurity. (submitted)

8. Chandrasekaran, L., He, C-Z., Krutzsch, H. C., Iruela-Arispe, M. L., and Roberts, D. D.: Modulation of endothelial cell behavior and angiogenesis by an α3β1 integrin-binding peptide from thrombospondin-1. (Submitted)

Abstracts

1. Guo, N., Krutzsch, H. C., Inman, J. K., and Roberts, D. D. Thrombospondin type 1 repeats specifically induce apoptosis of endothelial cells. The thrombospondin gene family and its functional relatives tenascins, osteopontin, and SPARC. Seattle, WA, 1996.

2. Roberts, D. D., Guo, N., Chandrasekara, L., Chandrasekaran, S., Inman, J. K., Krutzsch, H. C. Rolke of type 1 repeats in inhibition of breast carcinoma growth by thrombospondin-1. Era of Hope meeting, 1997.

3. Chandrasekaran, L., and Roberts, D. D. Mutation of anti-angiogenic sequences in the type 1 repeats of thrombospondin-1. Molec. Biol. Cell 9:298a, 1998.

Patents

A provisional U.S. Patent Application "α3β1 Integrin binding peptide from thrombospondin-1 and their uses" was filed on July 15, 1999.

Development of cell lines

We have developed a series of MDA MB 435 breast carcinoma-derived cell lines that stablely express various mutated thrombospondin-1 constructs. These will be made available to the scientific community following publication of a manuscript describing their properties.

Employment and research opportunities:

Mr. Rui Rodrigues received training supported by this grant and was a coauthor on the manuscript presented in Appendix D. Based in part on this training, he was accepted and is currently attending Medical School at Howard University.

Dr. S. Chandrasekaran received training supported by this grant and was the first author on the manuscript presented in Appendix ?. Based on this training, he was offered and accepted a position at Data Unlimited, Inc. which develops computer systems to assemble and analyze DNA data bases.

Dr Neng-hua Guo performed some of the initial work supported by this grant, although he did not receive salary support. He was first author on the two manuscripts presented in Appendices B and C and was a co-author on two other papers. After completing his

postdoctoral training, he accepted a Senior Staff Fellow position in the National Heart, Lung, and Blood Institute.

CONCLUSIONS

Development of stable peptide analogs to inhibit angiogenesis of breast cancers

We have examined two approaches to prepare angiogenesis inhibitors based on the type 1 repeats of TSP1. D-reverse analogs showed activity in both classical angiogenesis assays (chick CAM assay) and in a breast cancer xenograft model. Since these are small molecules with defined structure, they should be attractive lead compounds for drug development of therapeutic angiogenesis inhibitors. We will continue to develop the existing peptide analogs toward clinical application in collaboration with the NCI Developmental Therapeutics Program.

Our efforts to apply polymeric conjugates of the TSP1 peptides for breast cancer were unsuccessful. These conjugates are active in vitro and exhibit anti-angiogenic activity in a retinopathy of prematurity model. The larger size of these conjugates may aid their retention in the vitreous humor. These data demonstrate that different pathological conditions associated with angiogenic responses may differ in their response to specific angiogenesis inhibitors.

Functional analysis of anti-angiogenic sequences in thrombospondin-1

Full length thrombospondin-1 expression constructs containing four site-directed mutations of the type I repeat sequences have been prepared. These disrupt the WSXW motifs in each type 1 repeat and the latent TGFβ-activating sequence at the border of the first and second type 1 repeats. Stably transfected human breast carcinoma cell lines have been prepared expressing two of these mutants. Mutation of the central Trp residue in the second type I repeat resulted in reversal of the effect of TSP1 over expression on the tumorigenic potential of MDA-MB-435 cells. Suppression of tumorigenesis by expression of wild type TSP1 but not the W441A mutant was observed both in athymic nude mice and Beige XID mice, demonstrating that the anti-tumor activity of TSP1 does not require NK, B, or T cell responses by the host. Reversal of the anti-tumor activity of TSP1 following the W441A mutation is consistent with our hypothesis that the WSXW motifs play a role in the anti-tumor activity of thrombospondin but is not consistent with the recent report that binding of a different sequence in the type I repeats to CD36 mediates its anti-angiogenic activity (52). Based on transient expression, disruption of the WSXW motifs in the first but not the second or third type 1 repeats abolishes the ability of TSP1 to inhibit endothelial cell proliferation. The mutant TSP1 with the latent TGFβ-activating sequence disrupted, however, retains its antiproliferative activity for endothelial cells. Therefore, this sequence is not required for the antiproliferative effect of TSP1 on endothelial cells. Analysis of a truncated mutant TSP1 (WG1a) suggests that TSP1 fragments lacking the carboxyl-terminal domains may have increased anti-angiogenic activity.

Expression of the F432A mutant inhibits proliferation of endothelial cells in transient transfections, but the same mutant can not accumulate to sufficient levels to significantly

inhibit thymidine incorporation in transiently transfected breast carcinoma cells. This may explain our failure to isolate any stable MDA-MB-435 cell transfectants over-expressing this mutant transgene. F432A and W385A mutants are both properly folded and secreted in MDA-MB-435 cells. This contrasts with the mutants in the WSXW motif in the erythropoietin receptor, which was shown to be essential for delivery of this protein to the cell surface (41, 42). We will continue to examine the stability and fate of these mutant thrombospondins in the breast carcinoma cells.

Thrombospondin type I repeat mutants have been shown to inhibit proliferation of bovine aortic endothelial cells to different degrees. While the W385A mutant showed little to no inhibitory effect in our thymidine incorporation assay, both W441A and W498G showed 50-60% inhibition of cell proliferation. We were also unable to isolate stable clones that express the mutant W385A protein. In this regard we think that the W385A protein might be functioning in a way similar to the F432A mutant protein. We have made a truncation mutant of wild type TSP1, WG1a, which lacks the entire sequence 3' to the type I repeats. We have also shown that this mutant is a potent inhibitor of proliferation. Based on a recent report that gene therapy using a nonviral construct expressing a similar fragment of TSP1 in conjunction with p53 decreased breast tumor xenograft growth in nude mice (53), we will further examine the activities of this truncated TSP1.

We have reported in our earlier annual report that the F432A mutant protein is synthesized and secreted normally by the MDA-MB-435 cells. We had also reported that at 24 and 48 hours post transfection 50% and 30% cells compared to control were respectively positive for GFP protein, an indicator of cells that were transfected. So we had concluded that the mutant protein F432A is toxic to the MDA-MB-435 cells. Next, we wanted to see the effects of this mutant on incorporation of ^{3}H-thymidine, an indicator of cell proliferation. The TSP1 mutant F432A does not show a dose-dependent effect on inhibition of thymidine incorporation in MDA-MB-435 cells (Figure 1). When MDA-MB-435 cells are transfected with 24 μg of F432A DNA, the thymidine uptake stays at about 90% of control. This could mean that at higher concentrations the mutant F432A protein is toxic to the cells expressing it and the measured thymidine incorporation is that of untransfected cells. We have seen some inhibition of endothelial cell proliferation by the F432A mutant. In preliminary experiments, lower doses of this plasmid have produced stronger inhibition. This inverted dose-response suggests that transient transfection using lower doses of the F432A plasmid may allow us to examine the anti-proliferative activity of this mutant in both cell types.

Since it had been shown earlier that in MDA231 cells uPA and uPAR were up-regulated by thrombospondin (54), we wanted to see if TSP1expression had similar effects in MDA-MB-435 breast cancer cells. Compared to the parent cell line, stable over-expression of TSP1 was associated with a ten-fold increase in the uPAR level in the stable clone TH26, there was no significant change in the uPA level (Table 3). The interesting observation was that when MDA-MB-435 cells were transiently transfected with THBS-WT DNA or the empty vector, the uPAR level remained unchanged. Thus it seems that the increased uPAR level seen in the stable clone TH26 may not be directly due to the over-expression of thrombospondin.

Identification of the α3β1 integrin as a regulated TSP1 receptor on breast carcinoma cells

The α3β1 integrin mediates adhesion of MDA-MB-435 and MDA-MB-231 breast

carcinoma cells to TSP1. This β1 integrin is maintained in an inactive or partially active state in these cell lines but can be activated by exogenous stimuli including serum, insulin, IGF1 and ligation of CD98. In MDA-MB-231 cells, the inactive state of is maintained by a G-protein mediated signal, but this suppression can also be overcome by IGF1 receptor signaling. Stimuli that increase β1-dependent adhesion to TSP1 do not stimulate β3-dependent adhesion to TSP1, even though the cells express the known TSP1 receptor αvβ3 and this integrin is functional and inducible for vitronectin adhesion. It is notable that even within the breast carcinoma cell lines, pharmacological and physiological stimuli can differentially modulate activity of the α3β1 integrin for promoting adhesion or chemotaxis to TSP1. This finding implies a complex signaling process that regulates the recognition of pro-adhesive signals from TSP1 in the extracellular matrix. Both the IGF1 receptor and CD98 are components of this regulatory complex in breast carcinoma cells, but the mechanisms of their actions also remain to be defined.

CD98 was recently identified as an activator of β1 integrins by its ability to overcome Tac-β1 suppression of β1 integrin function (55, 56). Our data demonstrate that clustering of CD98 can also increase α3β1-mediated TSP1 interactions. This may simply result from clustering of the CD98-associated α3β1 integrin, which increases the avidity for cell adhesion to a surface coated with TSP1, or it may require specific signal transduction from CD98. Regulation of CD98 levels is probably responsible for the serum-induced increase in adhesion to TSP1, since serum increases CD98 surface expression in MDA-MB-435 cells. The insulin and IGF1-induced stimulation of TSP1 spreading and chemotaxis can not be explained by regulation of CD98 levels, however, since IGF1 down-regulates CD98 in these cells.

Only a small fraction of the α3β1 integrin on MDA-MB-231 and MCF-7 cells is constitutively active to mediate adhesion to TSP1. The inactive integrin appears to be on the cell surface, since it can be rapidly activated by the TS2/16 antibody or by IGF1 receptor ligands. The low basal activity of this integrin could be result from absence of an activator or expression of an inhibitor in MDA-MB-231 and MCF-7 cells. Several factors that suppress integrin function have been identified, including H-Ras (57), integrin-linked kinase, and protein kinase C (48). Additional proteins are known to associate with the α3β1 integrin, including some members of the TM4SF family and EMMPRIN (58, 59), but their roles in regulating function are unknown. In MDA-MB-231 cells, suppression of α3β1 appears to be an active process that can be disrupted by PT. Thus, a heterotrimeric G-protein signaling pathway appears to maintain MDA-MB-231 cells in an inactive state. This inhibitory pathway may also be specific for the α3β1 integrin in MDA-MB-231 cells, because unstimulated MDA-MB-231 cells can spread on type I collagen using α2β1 integrin. Unstimulated MDA-MB-435 cells show the opposite phenotype, with better α3β1-dependent adhesion to TSP1 than α2β1-dependent adhesion to collagen. The differential modulation of TSP1 interactions with these two cell lines by PT as well as the calcium ionophores demonstrates that regulation of α3β1 activity for TSP1 may differ even between two cell lines derived from the same type of human cancer.

The α3β1 integrin also plays a role in TSP1 interactions with endothelial cells

To date, several receptors for TSP1 have been described on endothelial cells, including the αvβ3 integrin, low density lipoprotein receptor related protein (LRP), proteoglycans, CD47, and CD36. A recent publication concluded that CD36 expression is necessary to

mediate an anti-angiogenic activity of TSP1 (52). Thus, we did not expect the α3β1 integrin to play a significant role in interactions of TSP1 with endothelial cells. However, using the TSP1 peptide ligand we identified for this integrin as well as specific blocking antibodies, we clearly demonstrated that the α3β1 integrin mediates spreading of endothelial cells on TSP1 and that engaging this integrin by TSP1 can induce proliferation of endothelial cells. By this mechanism, we show that TSP1 can have a pro-angiogenic activity and that the peptide that binds to this integrin can inhibit endothelial cell proliferation in vitro and angiogenesis in vivo, independent of CD36. This result suggests that this peptide could be used as a lead to develop a new class of angiogenesis inhibitors that act by disrupting α3β1 integrin function on endothelium.

Differential regulation of the α3β1 integrin in breast carcinoma and endothelial cells

Although IGF1 is a major regulator of the activation state of the α3β1 integrin in breast carcinoma cells, this growth factor did not activate the same integrin in endothelial cells. None of the growth factors we have tested could influence the activation state of endothelial cell α3β1. Rather, cell-cell contact appears to be the major regulator of activation in endothelial cells. Loss of cell-cell contact activates the α3β1 integrin to recognize TSP1, and restoration of contact inactivates the integrin. Loss of cell-cell contact must occur during neovascularization of a tumor. This regulation may therefore be important for understanding the control of tumor angiogenesis. Intact TSP1 inhibits angiogenesis in most contexts, but expression of some TSP1 fragments (10) or whole TSP1 in specific contexts can stimulate angiogenesis (60, 61). By understanding that TSP1 contains both anti- and pro-angiogenic sequences and identifying the respective receptors that mediate each response, we can both improve our molecular understanding of tumor angiogenesis and develop strategies to inhibit this essential step in breast cancer progression.

'So what' Section:

As a scientific product, this project has yielded a new understanding of how thrombospondin-1 acts both on endothelial and tumor cells. Identification of important receptors for thrombospondin-1 on breast cancer and endothelial cells and their differential regulation will help to define how thrombospondin-1 expression influences progression of breast cancer.

Based on our demonstration of efficacy in treating breast cancer xenografts in mice, this project could also yield a medical product. The peptides developed with support of this grant are currently under development at the NCI Developmental Therapeutics Program for application as therapeutic angiogenesis inhibitors. Through work with several collaborators, we have demonstrated their effectiveness as therapeutic angiogenesis inhibitors in animal models of three important diseases: glomerulonephritis (33), retinopathy of prematurity (Shafiee et al, manuscript submitted), and brain cancer (34). Thus, we are encouraged to continue exploring their utility in breast cancer.

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Regulation of tumor growth and metastasis by thrombospondin-1

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ABSTRACT **Thrombospondin-1 (TSP1) is an extracellular matrix glycoprotein that influences cell adhesion, motility, and growth. Based on its effects on tumor and endothelial cell behavior, this member of the thrombospondin gene family has attracted interest as a potential regulator of tumor growth and metastasis. Initial studies have confirmed that increased TSP1 expression suppresses growth or metastasis of some tumors in vivo and inhibits angiogenesis. These activities are cell type specific, however, since overexpression of TSP1 in some tumors causes increased tumor progression. One basis for these apparently conflicting observations may be the complexity of the protein. TSP1 interacts specifically with several cell-surface receptors, heparan sulfate proteoglycans, growth factors, and other matrix components. These multiple binding specificities, combined with the ability of TSP1 to activate latent transforming growth factor β and inhibit several proteases, suggest that exposure to TSP1 may initiate several intracellular signals. The integration of these signals may allow varied responses to TSP1. Furthermore, these signals may be received by the tumor cells, endothelial cells responsible for neovascularization, stromal cells, or cells of the host immune system. TSP1 influences specific behaviors of each cell type. Relating these phenomena to the molecular interactions of TSP1 observed in vitro may lead to novel therapeutic strategies for controlling cancer progression and metastasis.—Roberts, D. D. Regulation of tumor growth and metastasis by thrombospondin-1. *FASEB J.* 10, 1183–1191 (1996)**

Key Words: tumorigenesis · angiogenesis · cell-matrix interactions

GROWTH AND METASTASIS of tumors require many interactions between tumor cells and the host. The primary tumor must recruit a blood supply to obtain nutrients (1) and interact with the immune system in a manner that allows the tumor to avoid immune surveillance. A metastatic tumor must further accomplish a complex cascade of interactions with the host, including degradation of the extracellular matrix surrounding the primary tumor, invasion of tumor cells into blood vessels or lymphatics, homing to a target organ, extravasation, and migration through and degradation of matrix barriers in the target organs (2). These processes are mediated by specific expression in tumor cells of growth factors, motility factors, degradative enzymes, adhesion molecules, and specific receptors for each of these molecules. Tumor progression and metastasis also require loss of expression by the tumor cells of specific inhibitors of the growth factors, motility factors, and proteases and loss of specific adhesion molecules or their receptors that prevent tumor progression and invasion.

Adhesion molecules have attracted interest as potentially important regulators of tumor growth and metastasis after early observations that fibronectin is lost from the surface of some transformed cells. Manipulation of the expression of individual cell-matrix and cell-cell adhesion proteins in tumor cell lines (e.g., E-cadherin, N-CAM, syndecan, thrombospondin-1 [TSP1],[2] and some integrins) has been shown to be sufficient to suppress tumor growth or metastasis in animal models (reviewed in ref 3). Based on these results, efforts have been mounted to identify antagonists and modulators of adhesion molecules and to apply these materials as novel cancer therapeutics that may have fewer side effects than conventional cytotoxic agents.

A potential role for the extracellular matrix glycoprotein TSP1 in cancer is suggested by its regulated expression in many normal and tumor cells and by its ability to modulate adhesion, motility, or growth of many cell types in vitro (4, 5). TSP1 is the first identified member of the thrombospondin gene family (6). Because most data are available for TSP1 and the role of other thrombospondin family members in cancer is only beginning to be examined, this review will consider only the function of TSP1. Several recent reviews of the thrombospondins should be consulted for an overview of TSP1 functions (4, 5, 7) and the function and expression of other members of the gene family (4, 6, 8).

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[2]Abbreviations: TSP1, thrombospondin-1, product of the *THBS1* gene; TGF-β, transforming growth factor β; FGF-2, basic fibroblast growth factor; HSPG, heparan sulfate proteoglycan; IAP, integrin-associated protein or CD47; LRP, low-density lipoprotein receptor-related protein; NK, natural killer; PAI-1, plasminogen activator inhibitor-1.

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Both correlative data (9–14) and transfection experiments (15, 16) support the hypothesis that expression of TSP1 may decrease tumor growth or metastasis. However, conflicting results have been obtained (17, 18), and the effects of TSP1 appear to be specific both for the types of tumor examined and the experimental model used. These apparently conflicting data have led to some confusion about whether TSP1 should be classified as a tumor suppressor or as a promoter (15, 19, 20). Although much additional work is needed to define the net effect of endogenous TSP1 expression in tumors, we will attempt to define the effects of TSP1 on specific steps in the cascade of tumor progression (**Fig. 1**) and present evidence for the mechanism of these activities.

Understanding how cells respond to TSP1 is crucial to defining their responses to this protein. Signal transduction through interactions with extracellular matrix components is now recognized as an important regulator of cell growth and differentiated function (21). As is generally found in extracellular matrix proteins (22), TSP1 is a large protein with multiple structural domains and ligand binding sites (**Fig. 2**). This complexity predicts that TSP1 may simultaneously interact with more than one receptor on a single cell type and that the net response of a cell to TSP1 may reverse after a change in the relative expression of TSP1 receptors on the cell that signal positive or negative responses to a given pathway (4). Although it is premature to judge the net effect of these opposing signals in vivo, one can begin by examining the cell receptors for TSP1 and the effects of TSP1 on specific cellular responses. I will then propose ways that these biochemical effects of TSP1 can modulate host responses to a primary or metastatic tumor.

BINDING TO INTEGRINS AND INTEGRIN-ASSOCIATED PROTEIN

Cell adhesion to extracellular matrix is crucial to several steps in tumor progression and metastasis (Fig. 1). TSP1 contains an RGD integrin recognition sequence in the seventh type III repeat (Fig. 2). RGD-inhibitable adhesion to TSP1 has been demonstrated for cells expressing the integrin $\alpha v\beta 3$ (reviewed in ref 23). TSP1 also interacts with other integrins including $\alpha 3\beta 1$ on neuronal cells, $\alpha 4\beta 1$ and $\alpha 5\beta 1$ on activated T lymphocytes, and $\alpha IIb\beta 3$ and $\alpha 2\beta 1$ on platelets (24, 25; reviewed in ref 23). The sequences mediating interactions with integrins other than $\alpha v\beta 3$ have not been defined, but based on inhibition studies some are not RGD dependent. The cellular responses to TSP1 binding to specific integrins are only beginning to be explored. A report that TSP1 binding, mediated in part by the RGD-dependent integrin $\alpha v\beta 3$, stimulates transient calcium influx in IMR-90 fibroblasts (26) indicates that TSP1 can initiate signal transduction through integrin binding. Signaling through $\alpha v\beta 3$ may be relevant to regulation of angiogenesis by TSP1 because $\alpha v\beta 3$ plays an important role in regulating tumor angiogenesis (27).

Recently, an integrin-associated protein (IAP, CD47) was identified as the 52 kDa protein that binds to the adhesive motif VVM, expressed twice in the carboxy-terminal domains of TSP1 (28) (Fig. 2). Although IAP binds directly to TSP1 and VVM peptides, association of this protein with integrins, especially $\alpha v\beta 3$, suggests that adhesion of cells to TSP1 could involve a cooperative interaction of the VVM and RGD sequences with this protein complex. The conditions required for interaction of TSP1 with IAP remain puzzling, however, because IAP is expressed on almost all cell types, including erythrocytes, but TSP1 does not mediate adhesion of all cell types that express IAP. IAP binding through the VVM sequences may also elicit intracellular calcium signals (26). Overexpression in breast carcinoma cells of a carboxy-terminal deletion mutant of TSP1 lacking the second VVM motif reversed the suppressive effect observed using native TSP1 (15), suggesting that the VVM motifs play a role in

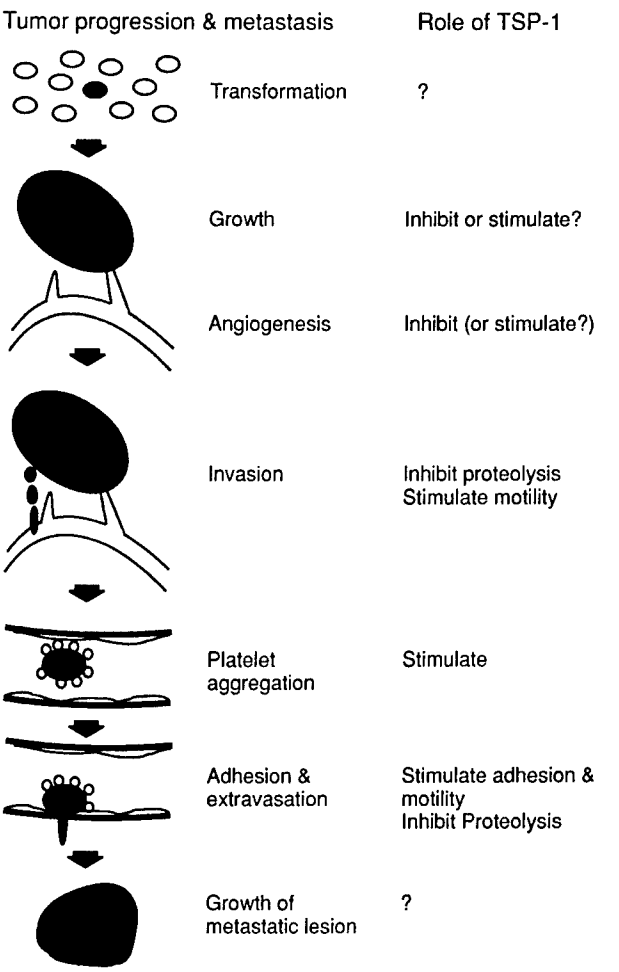

Figure 1. Potential role of thrombospondin-1 in tumor progression and metastasis. Steps in the cascade of tumor formation and development of metastatic lesions are depicted schematically showing potential sites of action of TSP1 to modulate the cascade.

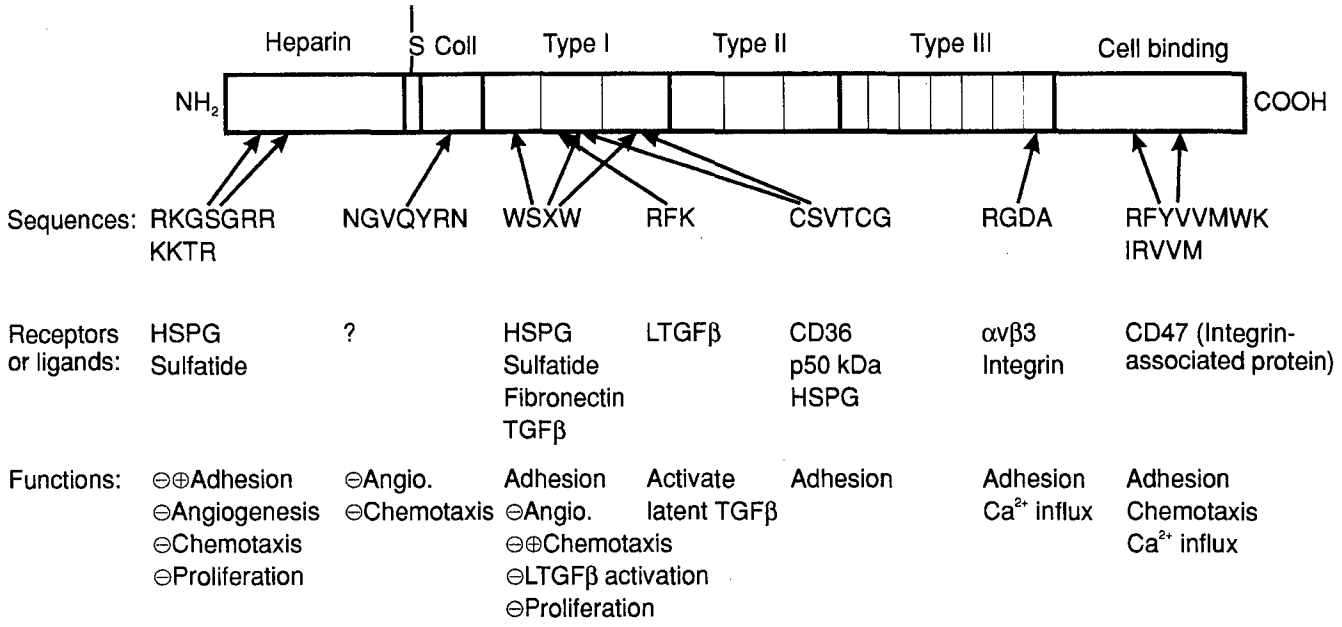

Figure 2. Structural and functional domains of thrombospondin-1. The diagram schematically depicts the organization of a single subunit of the TSP1 trimer, which is covalently assembled through disulfide bonds (S-). Functional peptide sequences identified from TSP1 are shown with the receptors or ligands recognized and the functional consequences of TSP1 binding to these ligands. Binding sites for other extracellular ligands and cell-surface receptors—including other integrins, LDL receptor-related protein, the 80/105 kDa receptor, fibrinogen, plasminogen activator, and neutral proteases—are not shown, as they are incompletely mapped.

the increased tumorigenesis of MDA435 breast carcinoma cells after overexpression of this mutant TSP1.

BINDING TO CD36 AND OTHER NON-INTEGRIN RECEPTORS

CD36 was the first nonintegrin receptor for TSP1 to be described (reviewed in ref 29). Purified CD36 binds to TSP1, and binding sites that may mediate this interaction have been identified on both proteins. Expression of CD36 on cells, however, is not always associated with an increase in TSP1 binding or adhesion, and specific conformation or phosphorylation states of CD36 may be required for TSP binding (29, 30). Nonetheless, several tumor cell lines expressing CD36 show partial dependence on this protein for adhesion to TSP1 (31), and this potential TSP1 receptor is expressed in some breast carcinomas (32). CD36 is a member of the class B scavenger receptor family. The low-density lipoprotein receptor-related protein (LRP) is another scavenger receptor that interacts with TSP1 (33, 34). LRP mediates rapid uptake and degradation of TSP1 by many cell types. The possible function of LRP in other cellular responses to TSP1 deserves further attention.

A novel receptor for TSP1 was identified on squamous carcinoma cells, composed of 80 and 105 kDa subunits (35). Although the size suggests an integrin receptor, the receptor was not immunologically related to known β1 or β3 integrins.

BINDING TO HEPARAN SULFATE PROTEOGLYCANS

TSP1 binds avidly to heparin, sulfatide, and HSPGs through an amino-terminal heparin binding domain (Fig. 2), through secondary heparin binding sites in the type I repeats, and possibly through additional undefined sites (reviewed in ref 36). This binding can allow TSP1 to interact with HSPGs in the extracellular matrix or on the cell surface, including syndecan. Because syndecan may be a signaling molecule and its expression influences tumor behavior, the effect of this interaction may be relevant to the role of TSP1 in cancer. A second effect of HSPG binding is to prevent FGF-2 and potentially other heparin-dependent growth factors, from binding to HSPG, which is essential for signal transduction by some of these growth factors. Thus, TSP1 can antagonize the stimulation of endothelial cell growth and motility stimulated by FGF-2 (37), and heparin binding recombinant fragments and peptides from TSP1 reproduce the FGF antagonist activity of intact TSP1 (38).

BINDING AND ACTIVATION OF LATENT TGF-β

TSP1 is a major glycoprotein in the α-granules of platelets and is secreted in response to platelet activation with several other α-granule components, including TGF-β. Some of the TGF-β binds tightly to TSP1 and is biologically active (39). Furthermore, addition of TSP1 to puri-

fied recombinant latent TGF-β results in its conversion to active TGF-β. This activation is probably mediated by the peptide sequence RFK in the type I repeats of TSP1 (Fig. 2), because peptides containing this sequence are equally as active on a molar basis as intact TSP1 for activating latent TGF-β (40). A second sequence adjacent to the activating sequence, WSHW, may play a role in binding of TGF-β to TSP1 and is an antagonist of activation mediated by purified TSP1. The RFK sequence is unique to TSP1, but the WSXW sequences are conserved in the type I repeats of thrombospondin-2. Synthetic peptides containing this sequence or recombinant thrombospondin-2 can antagonize the activation of latent TGF-β by purified TSP1 (40). Thus, TSP1 may be an activator of latent TGF-β, and thrombospondin-2 may be a negative regulator of this pathway of TGF-β activation.

At present, the physiological role of TSP1-dependent vs. protease-dependent or other pathways for activation of latent TGF-β is unclear. Several observations suggest that physiological activation of latent TGF-β by TSP1 may be regulated by additional factors. TSP1 overexpression in endothelial cells does not increase activation of endogenous latent TGF-β (16), and most TGF-β released from activated platelets remains in latent form despite exposure to high concentrations of platelet TSP1. Expression of α2-macroglobulin may also prevent activation of latent TGF-β by TSP1 (41).

REGULATION OF PROTEASES

Regulation of protease activity is required for tumor cell invasion and extravasation (Fig. 1). Several proteases, including cathepsin G, neutrophil elastase, plasmin, thrombin, and urokinase plasminogen activator, bind avidly to TSP1 (reviewed in ref 42). Complexes of TSP1 with the neutral proteases cathepsin G and neutrophil elastase result in competitive inhibition of activity. TSP1 is an inhibitor of plasmin and urokinase activity, but complexes of these enzymes with TSP1 may also be enzymatically active (43). Additional work is needed to define the conditions for inhibition and the role of TSP1 conformation and disulfide bond isomerization in the regulation of these activities.

Modulation of protease and protease inhibitor gene expression or secretion are also potential mechanisms for regulating extracellular protease activity by TSP1. Overexpression of TSP1 in transformed endothelial cells decreased net fibrinolytic activity due to increased plasminogen activator inhibitor 1 (PAI-1) and decreased urokinase secretion (16). Increased expression of PAI-1 was also observed in TSP1-treated endothelial cells (44). Although PAI-1 transcription is strongly induced by TGF-β, the increased secretion of PAI-1 observed in transfected cells overexpressing TSP1 was not associated with increased activation of latent TGF-β (16). Stimulation of

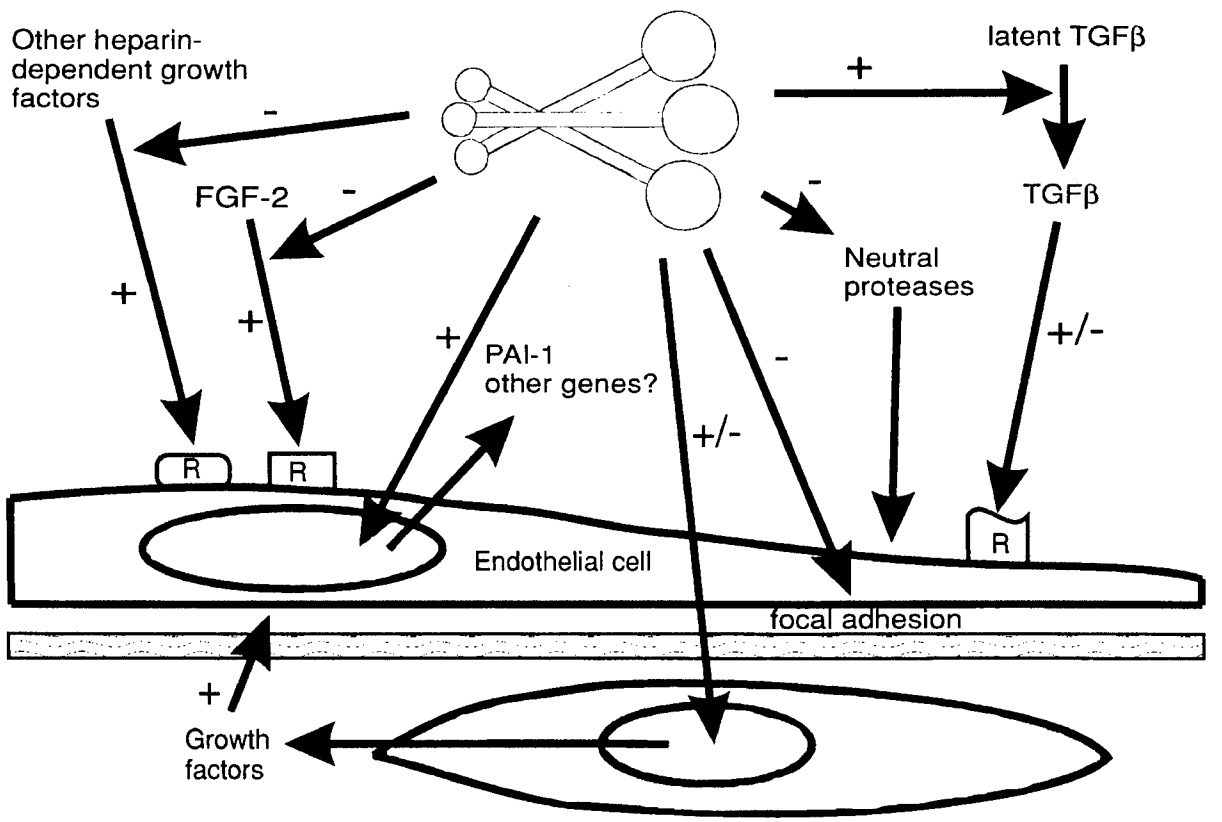

Figure 3. Pathways for regulation of angiogenesis by thrombospondin-1. Several pathways may account for the antiangiogenic activities of TSP1. TSP1 directly influences endothelial cell adhesion and expression of PAI-1, and indirectly influences endothelial cell behavior by antagonism of heparin-dependent growth factors, activation of latent TGF-β, inhibition of protease activity, and induction of growth factors in adjacent cells.

PAI-1 secretion may also account for the ability of TSP1 to modulate invasion of fibrin gels by A549 lung carcinoma cells and MDA231 breast carcinoma cells (43; reviewed in ref 20). The latter two activities were ascribed to TSP1-mediated TGF-β activation, but this hypothesis was not directly tested. Based on these data, however, TSP1 may regulate expression of protease and protease inhibitor genes both through direct and TGF-β-dependent pathways.

EXPRESSION OF TSP1 BY TUMOR CELLS

TSP1 is expressed by many types of tumor cells in vitro. In some cases, this endogenous TSP1 expression has been correlated with in vivo behavior. A positive correlation between TSP1 expression in vitro and tumor behavior in vivo was observed for a series of squamous carcinomas (45). In contrast, a negative correlation between TSP1 expression was observed for breast carcinoma cell lines (9, 10, 14), melanoma (9), *ras*-transfected human lung epithelial cells (9), and transformed endothelial cells (16).

Expression of TSP1 in tumor cells is regulated by several growth factors, oncogenes, and tumor suppressor genes including *p53, ras, c-jun, v-src*, TGF-β, platelet-derived growth factor, and FGF-2. A detailed discussion of the regulation of *THBS1* gene expression is beyond the scope of this review, but several recent reviews may be consulted for detailed discussions (4, 5, 8). Whereas changes in oncogenes and tumor suppressor genes can directly influence TSP1 expression in tumor cells, overexpression of growth factors by the tumor cells may also modulate TSP1 expression in stromal cells and in endothelial and smooth muscle cells of the tumor vasculature.

To understand the significance of the regulation of TSP1 expression observed in vitro, expression in vivo must also be examined. Expression of TSP1 in vivo has been studied most thoroughly for breast carcinoma (32, 46–48). Thrombospondin is synthesized by normal breast stromal cells in tissue culture (49) and is a normal component of human milk. Immunohistochemical analyses of TSP1 expression in malignant breast tissues demonstrated strong staining in desmoplastic stroma and in the basement membrane associated with malignant ductal epithelium (47). However, TSP1 is also expressed in the basement membrane of normal myoepithelial cells, and most invasive ductal carcinoma cells do not express TSP1 (32). High expression of TSP1 in invasive breast carcinoma is restricted to invasive lobular carcinoma (32). Migrating "Indian file" cells in invasive lobular carcinoma stain strongly for TSP1 (32, 48). Thus, expression of TSP1 may be selectively lost in invasive ductal carcinomas. In some tumors, increased expression of TSP1 was noted in stromal cells adjacent to tumor cells (47), but in most breast carcinomas secretion of TSP1 into the matrix by the tumor cells vs. stromal cells has not been established.

Data for TSP1 expression in most other tumor types are sparse. TSP1 and other adhesion molecule expression were examined in radial and vertical growth stages of primary melanomas and metastases (50). TSP1 and CD36 protein expression, detected in the cytoplasm of melanoma cells using antibodies, was not correlated with tumor progression or metastasis. Increased plasma TSP1 concentrations have been reported in malignancy, especially in gastrointestinal and lung cancers (20), but are also observed in several nonmalignant conditions. The source of the elevated plasma TSP1 in malignancy, however, has not been established.

EFFECTS ON TUMOR CELL BEHAVIOR

In vitro studies have defined effects of TSP1 on tumor cell adhesion, motility, protease activity, and proliferation. The effects of TSP1 on tumor cell interactions with matrix have generally been proadhesive and are mediated by interactions with integrin receptors, CD36, or HSPGs. Under specific conditions, however, TSP1 can also inhibit tumor cell adhesion (36). In addition to mediating cell-matrix adhesion, TSP1 may mediate cell-cell adhesion. Formation of microemboli consisting of tumor cells surrounded with a rosette of platelets (Fig. 1) is important for metastasis of some tumors to the lung (reviewed in 51). TSP1 has a positive effect on tumor platelet adhesion (52, 53). Adhesion of MCF-7 breast carcinoma cells to a monolayer of human umbilical vein endothelial cells may also be mediated in part by TSP1, because adhesion to a monolayer of endothelial cells was inhibited by soluble TSP1 or a polyclonal antibody against TSP1 (54). Although enhancement of cell-matrix and cell-cell adhesion is often associated with suppression of invasion and metastasis (3), the latter two activities of TSP1 suggest a positive effect of cell-surface TSP1 expression during the later stages of hematogenous metastasis (Fig. 1).

TSP1 has well-characterized effects on cell motility. TSP1 is both a chemotactic and haptotactic stimulator of tumor cell motility (11, 55). Motility of squamous carcinoma cells in a TSP1 gradient is correlated with their TSP1 expression and in vivo behavior (11). TSP1 enhanced lung cancer cell invasion through a fibrin matrix (43), but apparently due to changes in protease activity rather than direct effects on cell motility.

Altered TSP1 expression may influence behavior of tumor cells in animal models of tumorigenesis or metastasis. Overexpression of TSP1 in 3T3 mouse fibroblasts allowed anchorage-independent growth in soft agar, an indicator of transformation of 3T3 cells, but had no effect on tumorigenesis (18). This activity is reminiscent of the ability of TSP1 to support growth of NRK fibroblasts in soft agar by activating latent TGF-β. During tumor progression, however, many cancers lose sensitivity to growth modulation by TGF-β. A comparison of mouse melanoma lines of differing metastatic potential derived from the K1735 melanoma line demonstrated an inverse relation

between TSP1 expression in vitro and tumorigenic and metastatic behavior in vivo (9). A similar association was seen for human breast carcinoma cell lines differing in metastatic potential and for a series of cell lines derived from the immortalized lung epithelial cell line BEAS2B following transformation with three activated forms of *ras*. Passage of each of these lines through mice and reisolation of the cell lines from the tumors selected for cells with a further decrease in TSP1 expression at both the mRNA and protein level. Overexpression of TSP1 in transfected MDA435 breast carcinoma cells had no effect on tumor cell growth, colony formation in soft agar, or motility responses in vitro, but caused decreased tumorigenesis and metastasis in vivo. An inhibitory activity of TSP1 expression on tumorigenesis is supported by a study using aneuploid Li-Fraumeni fibroblasts, which contain a mutant *p53* allele (19). These cells become immortal and lose TSP1 expression. Transfection with wild-type *p53* restored TSP1 expression. TSP1 expression was also decreased after transformation of Chinese hamster ovary cells induced by nickel (12). Transcription of the *THBS1* promoter detected in a CAT reporter plasmid was strongly decreased in the nickel transformed cells but could be increased by cotransfection with an expression plasmid for the retinoblastoma tumor suppressor *Rb*. In the latter study, it is not clear whether loss of TSP1 expression is a cause or result of transformation, but the transfection experiments establish that increased TSP1 expression is sufficient to reverse the phenotype in vivo.

In contrast to these negative correlations, suppression of TSP1 expression in the highly metastatic squamous carcinoma cell line 11B using an anti-sense TSP1 cDNA suppressed their metastatic activity (17). Positive effects on experimental metastasis were also demonstrated when TSP1 was injected intravenously prior to injecting T241 sarcoma cells (20). This activity of TSP1 was lost in thrombocytopenic animals, suggesting that enhanced platelet interactions with tumor cells (Fig. 1) account for the observed effect of TSP1.

EFFECTS ON ANGIOGENESIS

Whether or not TSP1 expression by a tumor cell has a direct effect on behavior of a specific tumor cell type in vitro, the well-defined effects of TSP1 on endothelial cells (**Fig. 3**) may explain the suppressive effect on tumor growth of TSP1 expression observed in vivo in a spontaneous metastasis model (15). In this study, although transfection of MDA435 breast carcinoma cells with TSP1 did not alter any in vitro behavior of the tumor cells examined, growth in vivo was strongly suppressed (15). This suppression was dose dependent and correlated with reduction of angiogenesis in the tumors at early times.

Antiangiogenic activities of TSP1 have been demonstrated in several laboratories, and several mechanisms were proposed to account for this activity (Fig. 3). TSP1 inhibits adhesion of endothelial cells on a fibronectin matrix (56), suggesting that the loss of adhesion may inhibit endothelial proliferation. This effect is mediated by the amino-terminal, heparin binding domain of TSP1 (57). TSP1 also inhibits growth and motility of endothelial cells stimulated by FGF-2 by directly inhibiting growth factor binding (37, 38). Some of the growth suppressive activity of TSP1 purified from platelets may also be due to contamination with TGF-β (39), but activity of recombinant and synthetic peptides from TSP1 (38, 58) and transfection of endothelial cell lines with TSP1 (16) demonstrate that TSP1 contains intrinsic antiangiogenic activities. This is supported by the loss of TSP1 expression that accompanies conversion of endothelial cells to an angiogenic phenotype after transformation by polyoma virus, middle-sized tumor antigen (13). A direct role for TSP1 in regulating this phenotype is also supported by the observations that overexpression of TSP1 in stable transfectants derived from this line restores the normal phenotype in these cells (16) and that down-regulation of endogenous TSP1 expression in these cells using antisense TSP1 expression constructs increased chemotactic responses to FGF-2 and capillary morphogenesis in vitro (59). In addition to its effects on FGF-2 responses of endothelial cells, TSP1 expression may also regulate fibrinolytic activity of endothelial cells (Fig. 3). TSP1-transfected endothelial cells secreted less urokinase plasminogen activator and increased PAI-1 relative to controls (16).

The evidence for antiangiogenic activity of TSP1 in vitro is consistent with several reports of antiangiogenic activity of TSP1 in vivo (14–16, 58, 60). Although several laboratories have independently demonstrated antiangiogenic activities of TSP1, proangiogenic activity was also reported (61; reviewed in ref 20). In collagen or fibrin gels, addition of TSP1 increased angiogenesis and proliferation of an aortic ring culture (20). The cells that proliferated in response to TSP1 appeared to be myofibroblasts rather than endothelial cells, however, suggesting that the positive effect on angiogenesis was mediated by growth factors produced by the former cells. Similarly, the enhancement of lipopolysaccharide- or FGF-2-stimulated corneal angiogenesis in the presence of TSP1 was associated with increased influx of leukocytes, which may mediate the observed positive response (61). The basis for these conflicting results remains to be determined, but is reminiscent of the paradoxical activities reported for TGF-β in angiogenesis. TGF-β is a potent inhibitor of endothelial proliferation in vitro but can be both proangiogenic and antiangiogenic in vivo (62). Thus, both direct and TGF-β-mediated effects of TSP1 on angiogenesis may be bidirectional.

EFFECTS ON IMMUNE RESPONSES TO TUMORS

The second major requirement for tumor growth and metastasis is suppression of the host immune response. An-

titumor immunity is mediated by NK cell responses, cytotoxic T cell responses, monocytes, and tumoricidal macrophages. The first suggestion that TSP1 may influence the immune response came from the observation that TSP1 enhanced monocyte killing of squamous carcinoma (63). TSP1 stimulates monocyte motility and enhances oxidative response to the inflammatory agonist FMLP (reviewed in ref 64). Thus, TSP1 may be an important modulator of monocyte and neutrophil functions. TSP1 also promotes adhesion of both resting and activated T cells (25). Clearing of apoptotic neutrophils by macrophages is mediated by TSP1 and the receptors CD36 and $\alpha v\beta 3$ (65), suggesting that TSP1 may play a similar role in interactions of tumoricidal macrophages with tumor cells. However, not all macrophages or monocytes residing in a tumor are tumoricidal. Further work is needed to determine whether TSP1 expression by tumor cells can enhance or suppress tumoricidal activities of these cells.

The ability of TSP1 to activate latent TGF-β provides another mechanism by which TSP1 may modulate immune function. TGF-β is produced by many tumors and may exert an immunosuppressive effect on NK or other immune responses in breast cancer xenografts (66). Both TSP1 and TGF-β inhibit early proliferation of interleukin 2-stimulated NK cells (67), and the effects of TSP1 in this assay were reversed by blocking antibodies to TGF-β. This model, however, predicts that overexpression of TSP1 in breast carcinoma cells would lead to increased TGF-β activation in the vicinity of the tumor and allow increased tumor growth. Yet the opposite is observed (15). If activation of latent TGF by TSP1-independent pathways is adequate for immune suppression in this model, however, then further activation in TSP1 overexpressing cells may have no effect on immune function. Moreover, the effects of both TSP1 and TGF-β on NK proliferation are biphasic; in prolonged proliferation assays, NK expansion is enhanced by both proteins (67). The consequences of these changes in proliferation to tumor growth are unclear, because TSP1 had no effect on killing of target tumor cells by NK cells after either short or long term culture.

EFFECTS ON STROMAL CELLS

TSP1 enhances fibroblast proliferation (68), a major component of tumor stromal cells. Additional effects of TSP1 on gene expression in stromal cells need to be further examined. The promoter of *THBS1* is responsive to many growth factors and wound stimuli. The tumor environment provides many of these signals to stromal cells through secretion of growth factors and induces TSP1 expression. Stromal cells are the major site of induced TSP1 expression in endometrium stimulated by progesterone (69). TSP1 expression by the stromal cells may in turn influence growth of endothelium, immune responses, and behavior of tumor cells. Stromal cell TSP1 expression may therefore be an important mediator of growth factor signals produced by tumors and a source of feedback to the tumor from its environment.

CONCLUSIONS AND PROSPECTS

As has been demonstrated for several other adhesion molecules, modulating TSP1 expression can have profound effects on tumorigenesis and metastasis. Biological activities of TSP1 in vitro predict its ability to influence interactions of tumor cells with their extracellular matrix as well as to regulate stromal cell behavior, neovascularization, and host immune responses to the tumor. Although several laboratories have manipulated tumor growth by altering tumor cell expression of TSP1, the effect of tumor-derived factors on stromal and endothelial cell expression of TSP1 is more likely to represent the major control of TSP1 levels in most tumors. These interactions are complex, and the net effect of TSP1 to enhance or inhibit tumor progression will need to be defined for each tumor type.

The multifunctional nature of TSP1 presents a challenge for defining the mechanism for the many observed effects of TSP1 in tumor models. Its interactions with integrins and other surface receptors, proteases, and growth factors could each account for major effects on tumor behavior (Fig. 1). Activation of latent TGF-β presents a further complication because TGF-β itself has pleiotropic effects on cells, the direction and magnitude of which are themselves sensitive to the status and environment of the target cell. Other members of the TGF-β family remain to be tested for activation in the presence of TSP1 or other members of the thrombospondin family. Both of these tasks will benefit from the availability of TSP1 and TGF-β null mice and cell lines. Careful site-directed mutagenesis of TSP1 may also aid in the differentiation of these effector pathways.

The design of appropriate animal models presents an additional challenge for further examination of the role of TSP1 expression in tumorigenesis and metastasis. The site of implantation of tumor cells is important for tumorigenesis studies, and orthotopic models have been developed for some tumors to approximate the biological environment of a spontaneous primary tumor. However, surgical implantation of tumor cells or grafts places the tumor in a wound environment where stromal cell expression of TSP1 may be abnormally induced. The two common metastasis assays also assess different parts of the metastatic cascade (Fig. 1). Experimental metastasis, where tumor cells are introduced directly into the bloodstream, model only the latter portion of the cascade. The positive effects of exogenous TSP1 observed in some of these models are consistent with the observed effects of TSP1 on tumor cell interactions with endothelial cells and platelets. Spontaneous metastasis models depend more on the early events of metastasis, including angiogenesis. Thus, the antiangiogenic activities of TSP1 (Fig.

3) are consistent with observations of a negative effect of TSP1 overexpression on tumor growth in these models. Both metastasis models usually use aggressive tumor cell lines containing multiple genetic lesions. Yet the modulating effects of TSP1 may be more important during early development of a primary tumor (Fig. 1). Approaches must be developed to modulate TSP1 expression in premalignant lesions and to assess the expression of TSP1 during progression of these lesions.

At present, no mutant forms of TSP1 or rearrangements of the *THBS1* gene have been identified in tumors, and the available evidence does not support a role for *THBS1* as a classical oncogene or tumor suppressor gene. However, TSP1 expression is tightly regulated by several oncogenes and tumor suppressor genes and may play a significant role in expression of the malignant phenotype induced by alterations in these genes. Thus, it is reasonable to consider the possibility that pharmacological manipulation of TSP1 expression could control tumor growth or metastasis induced by these oncogenes.

TSP1 sequences that modulate growth factor activities or angiogenesis may be useful in modulating tumor growth or progression. Because small peptides derived from several regions of TSP1 mimic activities of the intact protein, these may serve as lead structures for synthesis of small molecules for therapeutic use. We have successfully used retro-inverso analogs of the TSP1 type I repeat peptides to systemically treat mice bearing breast carcinoma (N. Guo, H. C. Krutzsch, J. K. Inman, and D. D. Roberts, unpublished results). Peptide mimetics based on other regions of TSP1 should also be examined for efficacy in inhibiting tumor growth. Achieving a better understanding of the *THBS1* promoter and transcription factors that regulate its expression may lead to another therapeutic approach. Drugs may then be designed to directly regulate TSP1 expression in tumor stromal or vascular cells. Approaches such as these offer exciting opportunities to apply our expanding knowledge of cell-matrix interactions to cancer therapy. FJ

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Antiproliferative and antitumor activities of D-reverse peptides derived from the second type-1 repeat of thrombospondin-1

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The extracellular matrix glycoprotein thrombospondin-1 (TSP1) inhibits angiogenesis, endothelial cell growth, motility and adhesion. Peptides from the type I repeats of TSP1 mimic the adhesive and growth inhibitory activities of the intact protein and specifically interact with heparin and transforming growth factor-β (TGFβ). To define the structural basis for the antiangiogenic activities of these peptides, we prepared analogs of the TSP1 peptide KRFKQDGGWSHWSPWSSC. L-forward, L-reverse, and D-reverse (retro-inverso) analogs displayed identical activities for binding to heparin, demonstrating a lack of stereospecificity for heparin binding. The L-reverse and D-reverse peptides, however, had somewhat decreased abilities to activate latent TGFβ. Conjugation of the forward peptides through a C-terminal thioether and the reverse peptides through an N-terminal thioether to polysucrose abolished the adhesive activity of the peptides and enhanced their antiproliferative activities for endothelial and breast carcinoma cells stimulated by fibroblast growth factor-2. Their antiproliferative activities were independent of latent TGFβ activation, because substitution of an Ala residue for the essential Phe residue in the TSP1 type-1 repeat peptide increased their potency for inhibiting TSP1 binding to heparin and for inhibiting endothelial cell proliferation. Although the conjugated peptides were inactive *in vivo*, an unconjugated retro-inverso analog of the native TSP peptide inhibited breast tumor growth in a mouse xenograft model. Thus, these TSP-derived peptide analogs antagonize endothelial growth through their heparin-binding activity rather than through activation of latent TGFβ or increasing cell adhesion. These stable analogs may therefore be useful as therapeutic inhibitors of angiogenesis stimulated by fibroblast growth factor-2. © Munksgaard 1997.

Key words: thrombospondin-1; endothelial cells; adhesion; heparin-binding; transforming growth factor β; tumor growth inhibitors; D-reverse peptides

Growth of many solid tumors depends strongly on recruitment of neovascularization. Increased vascularization of primary breast tumors is associated with an increased rate of metastasis to lymph nodes and a poorer prognosis (1, 2). Angiogenesis is regulated by both stimulatory or angiogenic factors and inhibitory or antiangiogenic factors (1, 3). High expression of antiangiogenic factors and limited availability of angiogenic factors maintains the endothelium in a nonproliferative state. Pathological states such as wound repair, diabetic retinopathy or tumor growth may alter the balance of these stimulators or inhibitors to allow neovascularization to proceed (1, 3).

Several antiangiogenic factors have been identified, including thrombospondin-1 (TSP1) (4–7), interferon-α, platelet factor 4, SPARC (8), apolipoprotein E3 (9), angiostatin and a proteolytic fragment of fibronectin (1, 3). Some of these proteins bind to heparin, and this binding activity may be responsible in part for their antiangiogenic activities. We have recently shown that the heparin-binding protein apolipoprotein E3 and heparin-binding recombinant fragments and synthetic peptides from TSP1 compete for binding of FGF-2 to endothelial cells or heparin and thereby inhibit proliferative and migratory responses of endothelial cells to FGF-2 (9, 10).

TSP1 is a member of a family of extracellular matrix glycoproteins (11), and is released from platelets and secreted by many cell types *in vitro* (12, 13). TSP1 influences a complex array of biological responses (14),

Abbreviations: TSP, thrombospondin-1, product of the *THBS1* gene; TGFβ, transforming growth factor β1; FGF-2, basic fibroblast growth factor; AECM, aminoethylcarbamylmethylated; ri, retro-inverso; HBSS, Hanks' buffered salts solution; BSA, bovine serum albumin.

including cellular adhesion, proliferation and migration. These effects may be mediated by direct interactions of TSP1 with several cell-surface receptors. TSP1 may also act indirectly by interacting with other extracellular components such as heparan sulfate proteoglycans and neutral proteases and by modulating activities of the growth factors, TGFβ (15, 16) and FGF-2 (10).

Based on its effects on tumor cell adhesion, growth and motility, the expression of TSP1 in tumors could regulate their metastatic phenotype (17). We found that TSP1 mRNA and protein expression decreased in several tumor cell lines selected for high metastatic potential in mice (18). Expression of activating forms of the *ras* oncogene or loss of the wild-type tumor suppressor *p53* were also associated with loss of TSP1 expression (18, 19). Furthermore, overexpression of TSP1 in breast carcinoma cells suppressed tumor growth in nude mice (20), identifying *THBS1* as a potential tumor and metastasis suppressor gene. These data, combined with evidence that TSP1 inhibits endothelial cell growth and motility *in vitro* and angiogenesis *in vivo* (4–7, 21, 22), suggest that TSP1 may inhibit neovascularization of tumors.

Several sites on the TSP1 molecule may modulate angiogenesis. Two peptide sequences in the type I repeats, a peptide from the procollagen domain and a recombinant amino-terminal fragment of TSP1 inhibit endothelial growth or motility (10, 23). Synthetic peptides derived from the type I repeats of TSP1 inhibited proliferation and motility of endothelial cells stimulated by FGF-2, mimicking the activity of intact TSP1 (10). These peptides act at least in part by competing with FGF-2 for binding to heparan sulfate proteoglycan receptors on the endothelial cells, which are essential for presentation of FGF-2 to its signaling receptor (10). Although these synthetic peptides lack the contaminating TGFβ that may account for some antiangiogenic activities of platelet TSP1 (24), peptides from the second type I repeat contain the sequence RFK which activates purified latent TGFβ (16). The TSP1 peptide KRFKQDGGWSHWSPWSS, therefore, has three defined activities: promotion of adhesion, antagonism of FGF-2 and activation of latent TGFβ.

The strong antiproliferative activity of the TSP1 peptides suggested that they may be useful for inhibition of pathological angiogenesis *in vivo*. To achieve this goal, the multiple activities of the peptides must be resolved, and the role of each in the antiangiogenic activities of the TSP1 peptide must be defined. Furthermore, free peptides often have short half-lives in circulation because of their small size and susceptibility to proteolytic degradation. In several cases, use of polymer conjugates of peptides from extracellular matrix proteins has overcome these limitations (25, 26). Preparation of enzymatically stable retro-inverso analogs is a second method to increase the duration of activity of peptides *in vivo*. These analogs have been successfully applied to increase the stability and biological activity of peptide sequences for therapeutic applications (27, 28). Of particular relevance to the TSP1 peptides, an all D-amino acid peptide analog of the IKVAV peptide from the A chain of laminin replicated the activity of the natural sequence to influence tumor cell adhesion and growth *in vitro* and *in vivo* (29). Pseudo peptides based on the B1 chain of laminin also inhibited tumorigenesis and metastasis *in vivo* (30).

In this study, we have further examined the basis for the antiproliferative activity of these TSP1 peptides. Using analogs of the TSP1 sequence that separate the heparin-binding activity from the latent TGFβ-activating activity, we demonstrate here that only heparin-binding activity is required for antiproliferative activity. Using polymer conjugates of the peptides, we can also separate the adhesive activity of the peptides from their antiproliferative activities. A stereochemical analysis of the TSP1 peptides demonstrates that retro-inverso peptide analogs retain the heparin-binding activity. This observation allowed us to prepare proteolytically stable forms of the TSP1 peptides and to demonstrate their activity *in vivo* for inhibiting growth of a human breast carcinoma in an mouse orthotopic xenograft model.

EXPERIMENTAL PROCEDURES

Materials. TSP1 was purified from the supernatant of thrombin-stimulated human platelets (31). EGF and TGFβ1 were obtained from Gibco BRL (Gaithersburg, MD). Bovine aortic endothelial cells were used between passages 4 and 10. Normal rat kidney (NRK-49F), mink lung fibroblasts (Mv1Lu) and human breast carcinoma cells (MDA MB435) were obtained from the American Type Culture Collection (Rockville, MD). D-Amino acid precursors were obtained from Bachem (Torrance, CA). TSP1 and FGF-2 (Bachem) were iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) or Bolton-Hunter reagent (Dupont NEN, Boston, MA) as described previously (10).

Preparation of synthetic peptides. The peptides used in this study were synthesized on a Biosearch model 9600 peptide synthesizer using standard Merrifield solid-phase synthesis protocols and *t*-butoxycarbonyl chemistry (32). Peptides were analyzed by reverse-phase HPLC chromatography. Peptides for biological assays were further purified by dialysis using Spectrapor 500 M_r-cutoff membranes or by reverse-phase purification using C_{18} Sep-Pak cartridges. Identities of some peptides were verified by matrix assisted laser desorptive ionization time-of-flight mass spectrometry.

Preparation of polysucrose conjugates. Polysucrose with an average molecular weight of 400,000 or 70,000 (Ficoll, Pharmacia, Uppsala, Sweden) was first functionalized with primary amino groups as described previ-

ously (33, 34). This derivative, referred to as AECM-Ficoll and bearing 18–23 amino groups per 100 kDa (50 mg), was iodoacetylated in 1.35 ml of 0.15 M HEPES-NaOH buffer at pH 7.5 containing 1 mM EDTA by addition of 9.6 mg of iodoacetic acid *N*-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, MO) dissolved in 0.15 ml of dimethylformamide. After about 15 min of reaction, the solution was passed over a desalting column to obtain the iodoacetylated AECM-Ficoll. Nine micromoles of peptide were dissolved in 1.8 ml of distilled water, and 250 µl of a 50 mM solution of tris-(2-carboxyethyl)phosphine hydrochloride (Pierce Chemical) in water was added to the peptide solution, and the pH was adjusted to 7.1 to 7.8 by addition of 1 M Na_2CO_3. After 30 to 60 min, the resulting solution was passed through a column packed with 1.4 ml of Bio-Rad AG1-X8 anion-exchange resin in the acetate form. The column effluent was led into the iodoacetylated AECM-Ficoll solution, and the solution was stirred overnight at room temperature. The resulting solution was treated with 2-mercaptoethanol (20 mM for 1 h) and dialyzed against four changes of phosphate-buffered saline in a 12,000–14,000 M_r-cutoff tubing. The peptide concentration of the resulting conjugate was determined by measuring its absorbance at 280 nm using $\varepsilon = 5540\ M^{-1}\ cm^{-1}$ per Trp residue. The conjugates had between 8 and 29 moles of peptide covalently bound per mole of M_r 400,000 polysucrose.

Ligand binding assays. TSP1 binding to heparin-BSA was determined using a solid-phase assay (35). Heparin-BSA (0.075 µg/well) was adsorbed onto 96-well polyvinyl chloride microtiter plate wells in 50 µl of Dulbecco's PBS for 16 h at 4°C. After blocking with tris-BSA buffer (50 mM tris, pH 7.8, 110 mM NaCl, 0.1 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1% BSA), 30 µl of various concentrations of inhibitors diluted in tris-BSA buffer or buffer alone and 30 µl of ^{125}I-TSP1 (0.1–0.2 µg/ml) were added to each well. After incubation for 4 h at 4°C, the wells were washed, and the bound radioactivity was counted.

Binding of ^{125}I-FGF-2 to heparin was determined using an immobilized heparin-BSA conjugate as described previously (10). ^{125}I-FGF-2 was incubated with the inhibitors to be tested in heparin-BSA-coated wells for 2 h at 25°C. Bound radioactivity was determined after washing and cutting the wells from the plate.

Adsorption of peptides and polysucrose conjugates on plastic was quantified using a bicinchoninic acid reagent (Pierce Chemical) as described (36). The amount of adsorbed peptide was calculated using a standard curve constructed with the same peptide or conjugate.

Bioassay for inhibition of endothelial and breast carcinoma cell proliferation. Proliferation of bovine aortic endothelial cells was determined as described previously (10). Similar assays were performed using MDA MB435 human breast carcinoma cells except that the growth medium for the proliferation assays consisted of 5% fetal bovine serum in RPMI 1640 medium.

NRK fibroblast and mink lung cell bioassays for TGFβ were conducted as described previously (16, 37). Serum-free conditioned medium (37) prepared from bovine aortic endothelial cells at 70% confluency was used as a source of latent TGFβ for detecting activation by synthetic peptides in the NRK colony forming assay. NRK colonies in soft agar were quantified microscopically. Mink lung cell proliferation was determined in 96-well plates using 1×10^4 cells/well and quantified colorimetrically using the Abacus cell-proliferation assay (Clontech Laboratories, Inc., Palo Alto, CA).

Free and polysucrose-conjugated forms of peptide **476** (ri-amKRAKQAGGWSHWSPWSSCac) were submitted for testing in the National Cancer Institute Developmental Therapeutics Program *in vitro* screening cell panel. The peptides were tested for activity against 59 tumor cell lines in a 48-h proliferation assay as described (38).

Tumorigenesis assay in nude mice. NIH Nu/Nu mice, approximately 8 weeks of age, were injected in the mammary fat pads with 10^5 MDA MB435 cells. Six animals were injected for each condition per experiment. Beginning at day 25, the experimental animals were injected i.v. (tail vein) with 100 µl of the free peptide or polysucrose conjugates. Primary tumor size was determined twice weekly by length × width × height measurement. The presence of metastases was determined by gross autopsy and examination of hematoxylin and eosin-stained slides of step sections of the lungs and draining lymph nodes. The primary tumors were removed, stripped free of other tissues and weighed. Animal experiments were conducted in an AAALAC-accredited facility using a protocol approved by the NCI Animal Care and Use Committee.

RESULTS

Stereochemical specificity of heparin-binding and latent TGFβ activation. We previously reported that two Trp residues located no more than three residues apart are required for binding of the type I repeat peptides to heparin (35). To define the stereochemical specificity for this interaction, we prepared L-reverse and D-reverse analogs of the active peptide from the second type I repeat of TSP1 and tested their activity for inhibiting TSP1 or FGF-2 binding to heparin (Table 1). The modified L-reverse and D-reverse peptides **600** and **599** were equal in activity to the native L-forward peptide **246,** and all were more active for inhibiting TSP1 binding than for inhibiting FGF-2 binding to heparin (Table 1). As previously reported (16), the L-forward peptide **246** activated latent TGFβ, as assessed by stimulation of normal rat kidney fibroblast colony for-

TABLE 1
Stereospecificity for heparin-binding and TGFβ-activation by the TSP1 type I repeat peptide

Peptide	Sequence	TSP1 binding	FGF-2 binding	Latent TGFβ activation
246	L-KRFKQDGGWSHWSPWSS	0.17	1.3	0.002
600	L-SSWPSWHSWGGDQKFRK	0.14	0.9	0.03
599	D-SSWPSWHSWGGDQKFRK	0.12	0.9	0.01
596	D-SSWPSWHSWGGAQKARK	0.032	0.16	4
597	D-SSAPSAHSAGGDQKFRK	>10	>50	0.01
598	D-SSAPSAHSAGGAQKARK	>10	>50	>10

^{125}I-TSP1 or ^{125}I-FGF-2 and varying concentrations of peptides were added to microtiter plate wells coated with heparin-BSA, or with BSA to determine nonspecific binding, and incubated for 4 h at 4°C or 2 h at 25°C, respectively. ^{125}I-TSP1 or ^{125}I-FGF bound to the wells was quantified in a gamma counter. Results represent the micromolar concentration of peptide required for 50% inhibition of labeled thrombospondin-1 or FGF-2 binding to immobilized heparin-BSA determined from dose-response curves in at least two experiments. Latent TGFβ activation was determined by NRK colony formation in soft agar. Results are presented as the concentration of peptide giving 50% of maximal stimulation determined in two independent experiments.

mation in soft agar (Fig. 1). The L-reverse and D-reverse analogs also activated latent TGFβ (Fig. 1) but required higher concentrations than the L-forward peptide for half-maximal activation (Table 1). The dose for half-maximal stimulation by the L-forward peptide KRFKQDGGWSHWSPWSS (10 nM), measured

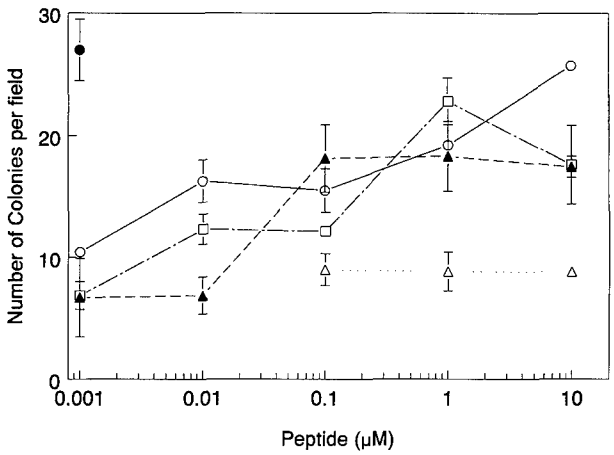

FIGURE 1
Stereochemical specificity for activation of latent TGFβ by peptide analogs of the second thrombospondin-1 type I repeat. The indicated concentrations of the L-forward peptide **246** (KRFKQDGGWSHWSPWSS, ○), the L-reverse peptide **600** (SSWPSWHSWGGDQKFRK, ▲), the D-reverse peptide **599** (ri-amKRFKQDGGWSHWSPWSSac, □) were mixed with serum-free conditioned medium from bovine aortic endothelial cell and added with NRK cells suspended in soft agar. The D-reverse peptide **598** (ri-amKRAKQAGGASHASPASSac, △) was used as a negative control, and conditioned medium heated to 85°C for 5 min to quantitatively activate the endogenous latent TGFβ was used as a positive control (●). Colony formation, determined as described under "Experimental Procedures," is presented as mean ± SD for triplicate determinations. Enhancement of colony formation by peptides 246, 599 and 600 was significant ($p < 0.05$ by a two-sided *t*-test) at 1 and 10 μM.

using endogenous latent TGFβ in endothelial cell conditioned medium, was higher than previously reported for the same peptide using purified latent TGFβ (16).

Separation of TGFβ-activating activity from heparin-binding. L-forward or D-reverse peptides lacking the Phe residue, shown previously to be essential for activation of latent TGFβ by the peptide KRFK (16), were weak or inactive for activating latent TGFβ in endothelial cell conditioned medium, based on the NRK colony formation assay (Fig. 1, Table 1). The active peptides at optimal concentrations stimulated 67% to 92% of the colony formation measured after complete activation of the latent TGFβ in the medium by heating at 85°C (Fig. 1). The D-reverse peptide **597,** with the three Trp residues substituted by Ala residues, activated latent TGFβ but lacked heparin-binding activity, as observed previously using the corresponding L-forward peptides (35). Conversely, the D-reverse peptide **596,** with the Phe residue substituted by Ala, had only weak TGFβ-activating activity but strongly inhibited heparin binding by TSP1 and FGF-2. The D-reverse peptide **598,** with five Ala substitutions, lacked all activity and was used as a negative control.

Separation of adhesive activity from antiproliferative activities. We attempted to use inhibition of mink lung epithelial cell proliferation (37) to confirm activation of latent TGFβ by the peptides. Although TGFβ inhibited growth of these cells, the retro-inverso peptide unexpectedly increased proliferation in a dose-dependent manner (Fig. 2A). This stimulation was observed in the absence of latent TGFβ in the medium. Identical stimulation was observed using a peptide lacking the RFK activation sequence (ri-amKRAKQAGGWSHWSPWSSCac), indicating that this effect is independent of the RFK motif. The stimulation of proliferation by the peptides was not specific to the Mv1Lu cells. Although some free TSP1 type

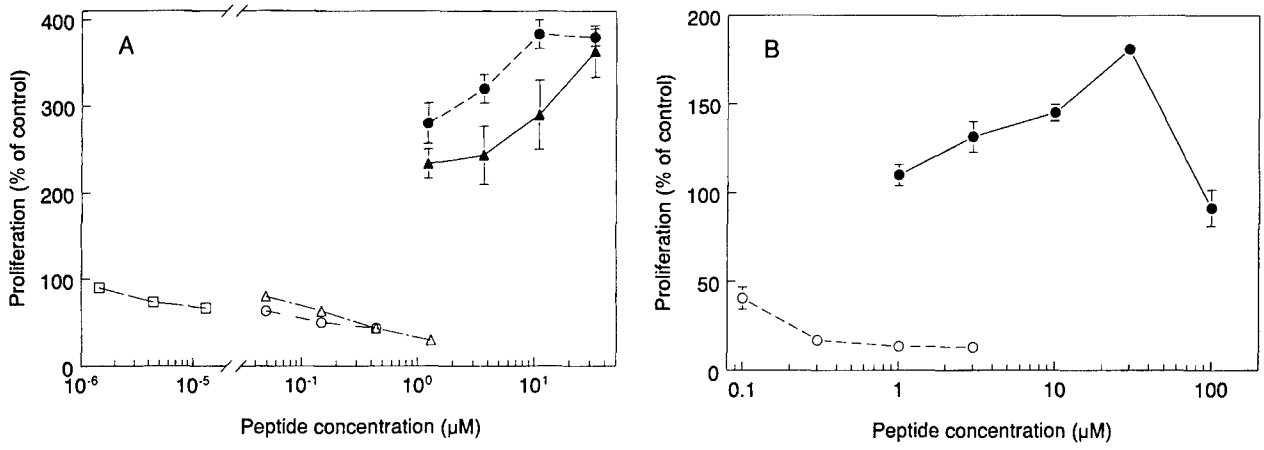

FIGURE 2

Conjugation of TSP1 peptides to polysucrose ablates growth stimulatory activity and increases their antiproliferative activity. (A) Effect of free and conjugated peptides on mink lung cell proliferation. Proliferation of Mv1Lu cells (1×10^4/well) was determined in the presence of the indicated concentrations of the TGFβ-activating peptide **416** (ri-amKRFKQDGGWSHWSPWSSCac, ●) or a polysucrose conjugate (○), peptide **476** lacking the activating sequence (ri-amKRAKQAGGWSHWSPWSSCac, ▲) or a polysucrose conjugate (△), or TGFβ (□). (B) Effect of free and conjugated peptides on endothelial cell proliferation. Proliferation of bovine aortic endothelial cells was determined in the presence of the indicated concentrations of peptide **416** (ri-amKRFKQDGGWSHWSPWSSCac, ●) or a polysucrose conjugate (○). Cell proliferation is presented (mean ± SD, $n = 3$) as a percent of that observed for cells grown in the same medium without additions.

I repeat peptides previously tested inhibited endothelial cell proliferation (10), several type I repeat peptides including the D-reverse peptide **416** also stimulated proliferation of endothelial cells at low concentrations (Fig. 2B and results not shown).

The strong adhesion-promoting activity of these peptides (32, 35) suggested that their positive effects on proliferation of mink lung and bovine endothelial cells resulted from this adhesive activity. The peptides that stimulated proliferation also promoted adhesion of Mv1Lu and BAE cells when adsorbed on plastic (Fig. 3). Polysucrose conjugates of the peptides, however, consistently did not stimulate cell adhesion (Fig. 3), and inhibited rather than stimulated mink lung cell (Fig. 2A) and endothelial cell proliferation (Fig. 2B). Furthermore, this inhibition was independent of latent TGFβ activation, because the peptide **476** conjugate, lacking the RFK motif, produced similar inhibition as the peptide **416** conjugate, containing the native TSP1 sequence (Fig. 2A). Polysucrose alone or polysucrose conjugated to control peptides was inactive. The lack of adhesive activities for the polysucrose conjugates probably results from their decreased adsorption on plastic. At the highest concentration used in these experiments, 40 μM, the adsorption of polysucrose conjugates (18–45 pmol/well) was only 10 to 20% of that for the corresponding free peptides (117–270 pmol/well). Conjugated peptides, therefore, separate the antiproliferative and adhesive activities of the peptides and were used to further study effects of the peptides on cell proliferation.

Specificity of the type I repeat sequences for antagonism of FGF-2 and TSP1 binding to heparin. Several L-forward peptides based on the type I repeats of TSP1 antagonized TSP1 or FGF-2 binding to heparin (Table 2). The ability to antagonize TSP1 or FGF-2 binding to heparin was specific to the TSP1 type I repeat peptides. We tested a peptide with known antiangiogenic

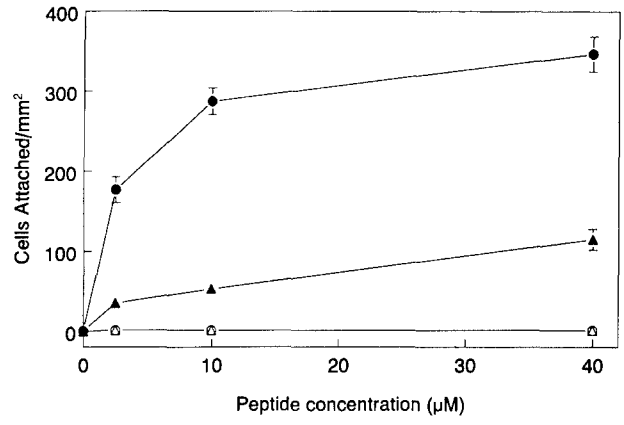

FIGURE 3.

Conjugation of TSP1 peptides to polysucrose ablates their ability to promote cell adhesion. Adhesion of Mv1Lu cells (●, ○) or bovine aortic endothelial cells (▲, △) to plastic coated with the indicated concentrations of TSP1 peptide **246** (KRFKQDGGWSHWSPWSS, ●, ▲) or a polysucrose conjugate of the peptide KRFKQDGGWSHWSPWSS (○, △) was determined. Results are the mean ± SD of triplicate determinations.

TABLE 2
Specific inhibition of thrombospondin-1 or FGF-2 binding to heparin-BSA by peptides derived from the type I repeats of thrombospondin-1

Peptide sequence	Heparin-binding protein	
	^{125}I-Thrombospondin IC_{50} (μM)[a]	^{125}(I-FGF-2 IC_{50} (μM)[a]
KRFKQDGGWSWHSPWSSC (TSP1)	0.5	3.8
KRFKQDGGWSHW	0.65	2.5
KRFKQDGG	>10	>40
SHWSPWSS	5	>40
TRIRQDGGWSHW (TSP2)	6	13
NGVQYRNCam (TSP1 procoll.)	>10	>40
FIRVVMYEGKK (TSP1 C-term.)	>10	>40
FRYVVMWK (TSP1 C-term.)		>40

^{125}I-TSP1 or ^{125}I-FGF-2 and varying concentrations of peptides were added to microtiter plate wells coated with heparin-BSA, or with BSA to determine nonspecific binding, and incubated for 4 h at 4°C or 2 h at 25°C, respectively. ^{125}I-TSP1 or ^{125}I-FGF bound to the wells was quantified in a gamma counter; am represents a C-terminal amide.
[a]IC_{50} values represent the concentration of peptide required for 50% inhibition of labeled thrombospondin-1 or FGF-2 binding to immobilized heparin-BSA.

activity derived from the procollagen domain of TSP1 (NGVQYRNC) (23) and two peptides with chemotactic activities for endothelial cells from the carboxyl-terminal cell binding domain of TSP1 (FIRVVMYEGKK or RFYVVMWK) (39, 40). Although these peptides contain basic amino acids and have a net positive charge at physiological pH, they did not inhibit FGF-2 binding to heparin (Table 2). A peptide from the second type I repeat of thrombospondin-2 (TRIRQDGGWSHW) homologous to the active TSP1 peptide (KRFKQDGGWSHW) inhibited FGF-2 binding, but was about 5-fold less active than the TSP1 peptide.

Activities for inhibiting FGF-2 binding to heparin differed in some cases from those determined using TSP1 as the labeled ligand. The basic amino acid motif and one of the Trp motifs were required for inhibiting FGF-2 binding (Table 2). Peptides containing only Trp motifs were uniformly inactive for inhibiting FGF-2 binding (SHWSPWSSC, Table 2; and SPWSEWTSCSTS and GPWSPWDICSVT from the first and third type I repeats of TSP1, results not shown), as were peptides containing the basic motif without the Trp motif (e.g., KRFKQDGG (Table 2) and KRFKQDGGASHASPASSC [Table 3]).

Substitution of the Asp residue at position 6 (peptide **392**) or the Gln and Asp residues at positions 5 and 6 by Ala residues (peptide **419**) improved the solubility of the peptides and also increased their heparin-binding activities (Table 3). Using the latter sequence, substitution of the Phe at position 3 by Ala to eliminate the TGFβ-activating activity of the TSP1 peptide (16) (peptide **450**), resulted in a peptide that retained the enhanced heparin-binding activity. The polysucrose conjugates of the peptides retained the heparin-binding activity of the free peptides (Table 3). Retro-inverso peptides with Ala substitutions showed a fairly direct correlation with the activities of L-forward peptides for inhibiting TSP1 binding. In general, the Ala substitutions increased the inhibitory activity and solubility. Retro-inverso peptides also showed similar activity as forward peptides for inhibiting FGF-2 binding to heparin (Table 3).

Inhibition of endothelial cell proliferation correlates with FGF-2 binding inhibition and does not require TGFβ activation. In general, the same structural requirements for antiproliferative activity were observed as when these peptides were tested for inhibition of heparin binding to FGF-2 (Fig. 4A and Table 3). Forward and retro-inverso peptide conjugates showed similar activities. Although TGFβ inhibited growth of the endothelial cells (Fig. 4A), the inhibitory activity of the peptide conjugates did not require the RFK sequence. A conjugate containing the native TSP1 sequence with Ala substitutions for Phe, Gln and Asp (peptide **450**) was highly active (Table 3), with an IC_{50} for inhibiting proliferation only 7-fold higher than that of native TSP1 (IC_{50} = 0.003 μM). This peptide was previously shown to not activate latent TGFβ (16). Endogenously produced latent TGFβ was not involved in the observed inhibition by the peptides, because an activating peptide (KRFK) had no effect on proliferation of the endothelial cells (Fig. 4A). A Trp motif was required for activity, as conjugates of forward or retro-inverso peptides lacking these residues (peptides 389, 475 and 513) were weak or inactive (Table 3). The basic motif also contributed to the antiproliferative activity as a conjugate of the peptide SHWSPWSSC had decreased activity (Fig. 4A). The inhibitory activity was specific for conjugates of the type 1 repeat peptides, as a

TABLE 3

Inhibition of thrombospondin or FGF-2 binding to heparin-BSA and endothelial cell proliferation by TSP1 peptide analogs

Peptide	Sequence	IC_{50} (μM)[a]			
		^{125}I-Thrombospondin binding to heparin		^{125}I-FGF-2 binding	BAE cell proliferation
		Peptide	Conjugate	Peptide	Conjugate
407	KRFKQDGGWSWHSPWSSC (TSP1)	0.5	3.5	3.8	0.5
392	KRFKQAGGWSHWSPWSSC	0.3	0.14	0.45	0.12
419	KRFKAAGGWSHWSPWSSCam	0.028	0.04	0.18	0.12
450	KRAKAAGGWSHWSPWSSC	0.03	0.025	0.22	0.02
389	KRFKQDGGASHASPASSC	N.D.	>5	>40	>10
500	NGVQYRNCam	>10	>10	>40	>10
416	ri-amKRFKQDGGWSHWSPWSSCac	0.4	1.0	2.8	0.13
474	ri-amKRAKQDGGWSHWSPWSSCac	0.1	0.3	0.95	N.D.
476	ri-amKRAKQAGGWSHWSPWSSCac	0.03	0.05	0.22	0.1
475	ri-amKRFKQDGGASHASPASSCac	10	>5	>20	4
513	ri-amKRAKQDGGASHASPASSCac	6.2	>5	18	>17

Labeled thrombospondin-1 or FGF-2 binding to immobilized heparin-BSA were determined as described in Table 1. BAE cell proliferation was determined in DMEM medium containing 0.5% FCS and 10 ng/ml FGF-2. IC_{50} values are the concentrations of peptide conjugates, expressed as molar concentrations of conjugated peptide, required for 50% inhibition of bovine aortic endothelial cell proliferation by the indicated derivatives of TSP1 peptides. Underlined residues indicate amino acid substitutions in the native sequence of the second type I repeat of TSP1. The retro-inverso peptides (ri) are shown C-terminus to N-terminus to facilitate comparisons with the L-forward peptides; ac represents an N-terminal acetyl, and am represents a C-terminal amide.

[a]IC_{50} values represent the concentrations of peptides required for 50% inhibition of net binding to immobilized heparin or proliferation of untreated BAE cells.

polysucrose conjugate containing the antiangiogenic procollagen domain peptide (NGVQYRNC) (23) was inactive (Table 3).

Inhibition of breast carcinoma cell proliferation. The peptide conjugates also inhibited proliferation of the human breast carcinoma cell line MDA MB435 (Fig. 4B). In contrast to the endothelial cells, growth of these cells was inhibited by TSP1 but not by TGFβ or by the free peptide KRFK, which activates latent TGFβ. In general, the peptide conjugates inhibited breast carcinoma cell proliferation to a lesser extent than endothe-

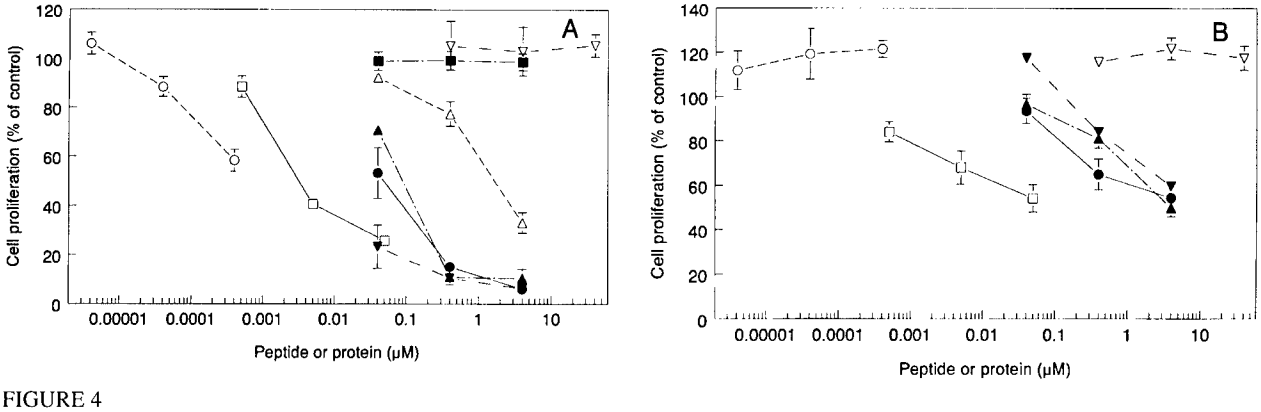

FIGURE 4

Inhibition of cell proliferation by thrombospondin peptide conjugates. (A) Inhibition of bovine aortic endothelial cell proliferation. Bovine aortic endothelial cells (5000/well) were plated with the indicated concentrations of peptides or conjugates in DMEM with 0.5% fetal calf serum and 10 ng/ml FGF-2. Proliferation was determined after 72 h as described under "Experimental Procedures." (B) Inhibition of breast carcinoma cell proliferation. MDA MB435 human breast carcinoma cells (10,000/well) were plated in wells containing the indicated concentrations of peptides or conjugates. Proliferation is presented as a percent of that determined in the absence of inhibitors, mean ± SD, n = 3, for the indicated concentrations of: thrombospondin-1 (□), TGFβ1 (○), a latent TGFβ-activating thrombospondin-1 peptide KRFK (▽), polysucrose control (■), or polysucrose conjugates of the peptides KRFKQDGGWSHWSPWSSC (●), SHWSPWSSC (△), acKRAKAAGGWSHWSPWSSCam (▼) and ri-amKRFKQDGGWSHWSPWSSCac (▲).

TABLE 4
In vitro screening of antitumor activities of TSP1 peptide analogs

Panel	Peptide **476**		Peptide **476**-polysucrose	
	Susceptible/tested	Mean IC_{50} (range)	Susceptible/tested	Mean IC_{50} (range)
Leukemia	6/6	20 (10–39)	3/6	2.5 (1.0–5.3)
NSCLC	4/9	19 (15–28)	0/9	
Colon	5/6	25 (11–41)	2/6	4.0 (2.3–5.8)
CNS cancer	4/6	15 (14–17)	0/6	
Melanoma	8/8	19 (10–46)	3/8	3.4 (1.0–6.0)
Ovarian	1/6	43	1/6	2.4
Renal	5/8	18 (10–43)	0/8	
Prostate	1/2	17	0/2	
Breast	7/8	15 (8–29)	3/8	2.1 (1.1–2.7)

Free and polysucrose-conjugated forms of peptide **476** (ri-amKRAKQAGGWSHWSPWSSCac) were tested for activity against 59 tumor cell lines in a 48-h proliferation assay as described (38). The number of cell lines showing >50% inhibition of proliferation at the highest dose tested (50 μM free peptide or 6.7 μM peptide as conjugate) is presented. For the susceptible cell lines, the mean and range for the IC_{50} values are presented.

lial cell proliferation, but the doses required for half-maximal inhibition were similar for the two cell types.

Spectrum of tumor cell growth inhibition. Inhibition of tumor cell growth by the peptides was not restricted to MDA MB435 cells. The peptide **476** (ri-amKRAKQAGGWSHWSPWSSCac) significantly inhibited growth of many tumor cell lines in the NCI Developmental Therapeutics Program *in vitro* panel (Table 4). As expected, the polysucrose conjugate was more active than the free peptide. Susceptibility varied among the panels, with breast cancer, leukemia and melanoma cell lines showing the broadest inhibition and ovarian cancer lines being generally resistant to growth inhibition.

Effect of peptides on tumor growth. A retro-inverso analog of the native TSP1 sequence, peptide **416**, was tested for inhibition of MDA MB435 tumor growth in an orthotopic nude mouse model. Growth of this tumor was previously demonstrated to be inhibited by overexpression of a TSP1 cDNA in the tumor cells, which was associated with reduced angiogenesis of the tumors (20). Tumor cells were allowed to implant for approximately 3 weeks before administering the peptide analog or a polysucrose conjugate systemically. No significant inhibition of tumor growth was observed in the animals treated with 5.3 mg/kg of 400,000 M_r polysucrose containing 0.2 mg/kg of bound peptide (Fig. 5) or in three additional experiments using daily injections of up to 0.5 mg/kg bound peptides as polysucrose conjugates (results not shown).

In contrast to the conjugate, daily intravenous treatment with the free peptide **416**, ri-amKRFKQDGGWSHWSPWSSCac, at 2 mg/kg significantly inhibited tumor growth (Fig. 6, A and B). Administration of the peptide before the tumor became palpable produced greater inhibition of growth (Fig. 6A) than when the peptide was administered after the tumor became palpable (Fig. 6B). The growth inhibitory effect persisted beyond the treatment period, although the growth of the tumors eventually resumed in most animals. At the time of sacrifice, the treated animals in the experiment shown in Fig. 6A had significantly smaller tumors 200 ± 275 mg (mean ± SD) than those in the control group 754 ± 228 mg, p = 0.03 by two-sided *t*-test. Histological examination showed increased infiltration of the treated tumors with mononuclear cells. Five of six animals treated in the first experiment had viable tumor cells on histological examination, and the sixth showed only fibrotic tissue with no evidence of viable tumor cells. A dose response using 0.2–6 mg/kg of the pep-

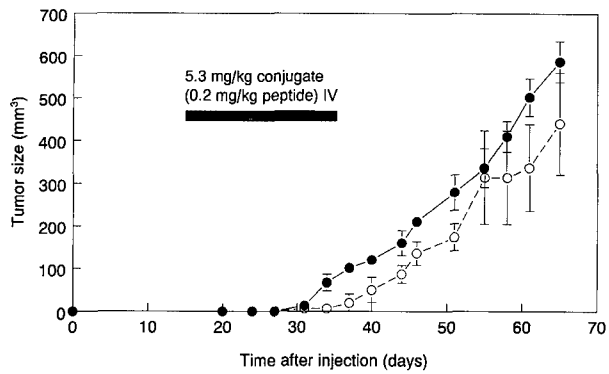

FIGURE 5
Effect of a polysucrose conjugate of the thrombospondin-1 mimetic ri-amKRFKQDGGWSHWSPWSSCac on growth of MDA MB435 human breast carcinoma xenografts in athymic nude mice. Tumor dimensions, determined by external caliper measurements, are presented as a function of time after implantation in the mammary fat pad. Results are the mean ± SEM for each group of six animals treated with the 0.2 mg/kg of the conjugated peptide (open circles) or mock treated by injection of HBSS vehicle (closed circles).

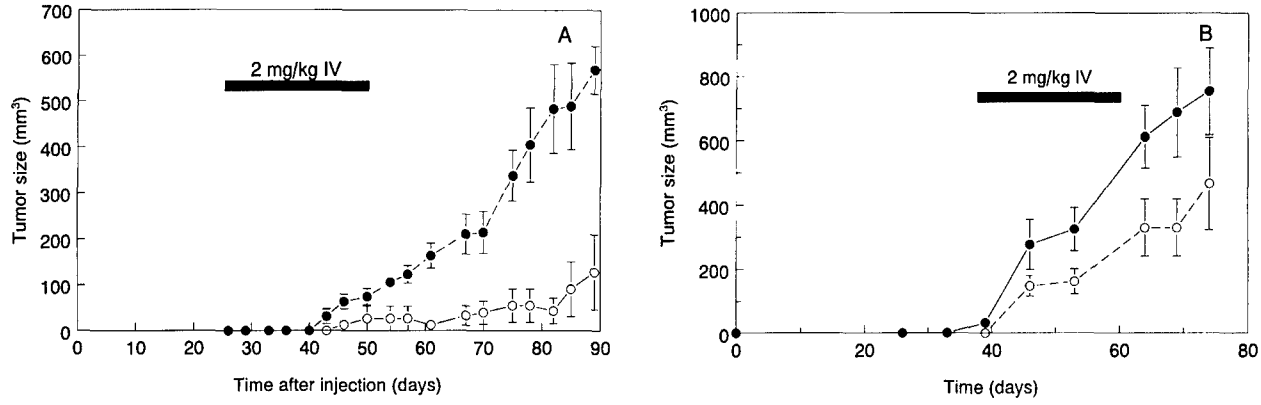

FIGURE 6

Inhibition of MDA MB435 tumor xenograft growth in nude mice by the thrombospondin-1 mimetic ri-amKRFKQDGGWSHWSPWSSCac. Tumor dimensions, determined by external caliper measurements, are presented as a function of time after implantation. Animals were treated by intravenous injection of 2 mg/kg of the peptide in HBSS or a corresponding volume of HBSS daily on days 25–50 (A) or days 35–60 after implantation of the tumor cells (B). The mean tumor volume ± SEM is presented for groups five to six animals treated with peptides (open circles) or mock-treated by injection of HBSS vehicle (closed circles). The animals were sacrificed on day 101, and the tumors were excised and weighed: control group, 754 ± 228 mg; treated group, 200 ± 275 mg (mean ± SD).

tide showed maximal inhibition of tumor growth after treatment with 6 mg/kg of peptide (Fig. 7). Animals were treated with up to 10 mg/kg without overt toxicity, based on relative weight gain in the control and treated groups and gross necropsy performed 10 days after termination of treatment.

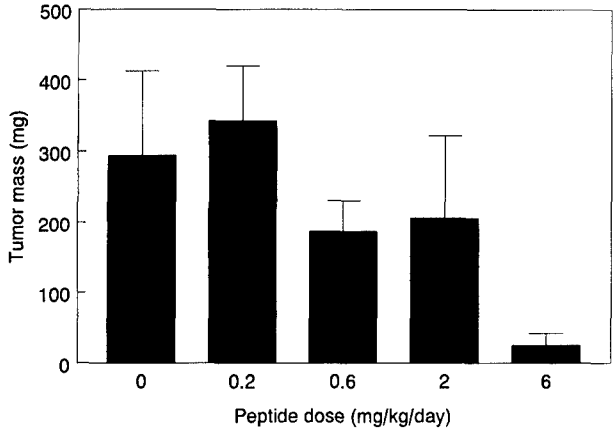

FIGURE 7

Dose dependence for inhibition of MDA MB435 xenograft growth in nude mice by intravenous treatment with the thrombospondin mimetic ri-amKRFKQDGGWSHWSPWSSCac. Animals were implanted with 10^5 MDA 435 cells in one mammary fat pad on day 0. Treated animals were injected daily by tail-vein injection with the indicated doses of peptide dissolved in 0.1 ml of HBSS from day 25 to day 50. Control animals were injected with HBSS alone. Animals were sacrificed on day 67. Tumors were excised and weighed. Results are presented as mean ± SD, $n = 6$. At the 0.6, 2 and 6 mg/kg doses, the p values for the observed inhibition were 0.07, 0.2 and 0.002, respectively, using a two-sided t-test.

DISCUSSION

Based on their ability to inhibit FGF-2 binding to heparin and endothelial cells (10), the antiproliferative activity of the type I repeat peptides from the second type-1 repeat of TSP1 result from antagonism of heparin-dependent growth factors such as FGF-2. This mechanism is supported by the similar activities of the L-forward and retro-inverso peptides for inhibiting FGF-2 binding to heparin and FGF-2-stimulated endothelial cell proliferation. Ablating the adhesive activity of the peptides by conjugation to a soluble polymer improved their antiproliferative activity *in vitro,* but only the free peptide significantly inhibited growth of a human breast carcinoma in a nude mouse xenograft model. The retro-inverso analog of the native TSP1 sequence reproduced the antiproliferative activity of TSP1 overexpression in this same xenograft model (20). Heparin-binding and antiproliferative activities of the peptides were coordinately increased by substitution of Ala residues at certain positions in both types of peptides.

The antiproliferative activity of the TSP1 peptide analogs *in vitro* does not require the TGFβ-activating sequence KRFK (16), because pepides containing the inactive sequence KRAK have similar inhibitory activities in endothelial, breast carcinoma and fibroblast proliferation assays. These peptides still contain the WSHW motif that was shown to inhibit TGFβ activation mediated by TSP1 (16) and which could result in inhibition of endothelial cell proliferation in the presence of latent TGFβ (15). Lack of growth inhibition by the activating peptide KRFK, however, suggests that sufficient latent TGFβ is not produced by the endothelial or breast carcinoma cells to account for the observed inhibition. Furthermore, growth of the breast

carcinoma cells was not sensitive to TGFβ. Therefore, the antiproliferative activities of TSP1 and the TSP1 peptides in these cells is clearly independent of latent TGFβ activation.

The heparin-binding activity of the type I repeat peptides is not stereospecific, whereas activation of latent TGFβ showed a moderate degree of stereospecificity. For the latter activity, L-reverse peptides were less active than D-reverse analogs, which share the same stereochemistry for the aminoacyl side chains as the native L-forward sequence. D-reverse peptidomimetics that retain activity and are stable to enzymatic degradation *in vivo* were developed based on this information. Two classes of stable analogs of the TSP1 type I repeat sequence have been prepared. Retro-inverso peptides lack peptide bonds sensitive to proteases but retain the heparin-binding and some of the latent TGFβ-activating activities of the native sequence. These peptides also exhibit antitumor activity *in vivo.* Polysucrose conjugates of the forward and retro-inverso peptides displayed increased antiproliferative activities *in vitro* and lack the adhesive activity of the free peptides. Although the polysucrose conjugates of these peptides could have longer circulatory half-lives than the free peptides *in vivo,* (25), these analogs were not active in the breast tumor growth assay. At present we can not distinguish whether this is caused by decreased ability of these larger molecules to diffuse into the tumor tissues or is related to the differences in cell adhesive activity observed *in vitro.*

Amino acid substitutions that removed latent TGFβ-activating activity coincidentally increased heparin-binding activity of the peptide mimetics. Substitutions of Ala residues for several amino acid residues in the native TSP1 sequence increased heparin-binding activity up to 20-fold, based on inhibition of TSP1 or FGF-2 binding. It remains to be determined whether the enhancement of activity resulting from these aminoacyl substitutions is direct or due to stabilization of a preferred binding conformation of the essential side chains. Ala residues may stabilize an α-helical conformation of the peptides. Preliminary data suggest that part of the enhancement is caused by changes in the aggregation state of the peptides (Krutzsch, H.C., Guo, N., King, C., Inman, J.K., and Robert, D.D., manuscript in preparation). The ability of some WSXW peptides to inhibit TSP1 binding but not FGF-2 binding suggest that FGF-2 and TSP1 bind to different determinants on heparin. The WSXW motif is sufficient to inhibit binding of TSP1, whereas basic residues are also required for inhibition of FGF-2 binding. These data are consistent with analyses of the heparin-binding specificities of the type I repeat peptides, showing overlapping but distinct binding specificities for the TSP1 peptides and FGF-2 (Yu, H., Tyrrell, D., Guo, N., and Roberts, D.D., manuscript in preparation). This conclusion is also supported by the significant difference in potencies of the peptides determined using the two heparin-binding proteins.

Although other peptides in the type I repeats and a peptide from the procollagen domain inhibit angiogenesis (23), their mechanisms of action are probably different from that of the present peptides. The procollagen domain peptide used in the present experiments did not inhibit FGF-2 binding to heparin or growth of endothelial cells. A peptide from the second type I repeat of thrombospondin-2 homologous to the active TSP1 peptides, however, also inhibited FGF-2 binding to heparin. This active sequence may account for the recently reported antiangiogenic activity of thrombospondin-2 (41).

Peptides containing D-amino acids often have increased stability in biological fluids because of their resistance to enzymatic degradation (42, 43). Although a D-amino acid homolog of the laminin peptide IKVAV retained activity (29), D-forward peptides present an inverted configuration that may not be bind to a receptor that recognizes the mirror image L-forward sequence (42). D-reverse and modified retro-inverso peptides, in contrast, present the same configuration of amino acid side chains as the corresponding L-forward peptide. With appropriate charge modifications of the terminal residues, these peptidomimetics retain activity unless specific peptide backbone interactions are required (27).

Activity of the TSP1 retro-inverso analogs demonstrates that the aminoacyl side chains are the major contributors to the heparin-binding and TGFβ-activating activities of the TSP1 peptide and that specific interactions with the peptide backbone or terminal charges of the unmodified peptides are not crucial. These results suggest that additional nonpeptide mimetics with potent heparin-binding activity could be prepared based on the aminoacyl substituents in the active peptides.

The mechanism of the antitumor activity of the peptides in the mouse xenograft model remains unclear. Inhibition of tumor growth *in vivo* may depend on antagonism of FGF-2 responses as observed *in vitro,* or may result from modulation of latent TGFβ activation because of the activating and inhibiting sequences present in the peptide used for these experiments. Based on *in vitro* proliferation data, the peptides have some direct effect on MDA 435 cell proliferation and a stronger inhibitory effect on endothelial cell proliferation. These peptides also selectively induce apoptosis of endothelial cells (44). Thus, endothelial cells are probably the main target for inhibiting tumor proliferation. Increased infiltration of the tumors by monocytes was consistently observed in mice treated with the retro-inverso TSP1 peptide. Because the athymic mice used retain some B and NK cell functions and have functional macrophages and monocytes, modulation of host immune responses to the tumor may also play some role in the action of the peptides. TSP1 could modulate NK cell function in the mice through regulating their expansion (45), although effects on target cell kill-

ing have not been observed. TSP1 may also regulate recognition of tumor cells by monocytes (46). Effects of the TSP1 peptides on these or other aspects of tumor immunity have not been reported, but will require further examination.

The greater activity of the polysucrose conjugates to inhibit endothelial cell proliferation *in vitro* did not predict activity *in vivo*. A limited volume of distribution of the larger peptide conjugates may limit their access to the presumed site of action of the peptides, inhibiting angiogenic responses of endothelium to FGF-2 from the tumor diffusing through the subendothelial matrix. Our data are consistent with the hypothesis that the peptides act by inhibiting neovascularization of the growing tumors stimulated by heparin-dependent growth factors such as FGF-2. The temporary inhibition of tumor growth observed using a retro-inverso peptide is consistent with the known effects of other antiangiogenic agents *in vivo* (1). These stable analogs of the TSP1 peptides therefore merit further development as therapeutic inhibitors of angiogenesis. Based on their ability to inhibit growth of some breast cancer, leukemia and melanoma cell lines *in vitro,* the peptides may also be useful for directly modulating growth of these tumors.

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Thrombospondin 1 and Type I Repeat Peptides of Thrombospondin 1 Specifically Induce Apoptosis of Endothelial Cells[1]

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ABSTRACT

Thrombospondin 1 (TSP1) inhibits angiogenesis and modulates endothelial cell adhesion, motility, and growth. The antiproliferative activity of TSP1 is mimicked by synthetic peptides derived from the type I repeats of TSP1 that antagonize fibroblast growth factor 2 and activate latent transforming growth factor β. These TSP1 analogues induced programmed cell death in bovine aortic endothelial cells based on morphological changes, assessment of DNA fragmentation, and internucleosomal DNA cleavage. Intact TSP1 also induced DNA fragmentation. The endothelial cell response was specific because no DNA fragmentation was induced in MDA-MB-435S breast carcinoma cells, although TSP1 and the peptide conjugates inhibited the growth of both cell types. Apoptosis did not depend on activation of latent transforming growth factor β because peptides lacking the activating sequence RFK were active. Apoptosis was not sensitive to inhibitors of ceramide generation but was inhibited by the phosphatase inhibitor vanadate. Induction of DNA fragmentation by the peptides was decreased when endothelial cell cultures reached confluence. Growth of the cells on a fibronectin substrate also suppressed induction of apoptosis by TSP1 or the peptides. Differential sensitivities to kinase inhibitors suggest that apoptosis and inhibition of proliferation are mediated by distinct signal transduction pathways. These results demonstrate that induction of apoptosis by the TSP1 analogues is not a general cytotoxic effect and is conditional on a lack of strong survival-promoting signals, such as those provided by a fibronectin matrix. The antitumor activity of TSP1 may therefore result from an increased sensitivity to apoptosis in endothelial cells adjacent to a provisional matrix during formation of vascular beds in tumors expressing TSP1.

INTRODUCTION

The extracellular matrix provides both positive and negative signals to regulate endothelial cell growth. Growth factors such as FGF-2[3] and vascular endothelial cell growth factor promote the growth and survival of nontransformed endothelial cells only when the cells are adherent to an appropriate extracellular matrix. Fibronectin is one of the matrix proteins that provides these signals. Fibronectin is an important matrix component for promoting the survival and growth of many cell types. The binding of fibronectin to the integrin receptor $\alpha_5\beta_1$ induces activation of signal transduction pathways including the focal adhesion kinase (reviewed in Refs. 1 and 2) and other protein kinases (3–5) and results in signals that maintain viability, such as up-regulation of Bcl-2 in Chinese hamster ovary cells (6) and mitogen-activated protein kinase in fibroblasts (7). The absence of appropriate matrix signals can induce programmed cell death or apoptosis of endothelial cells (8, 9). Inhibitors of a second integrin, $\alpha v\beta 3$, also induce apoptosis of angiogenic blood vessels and regression of tumors dependent on this neovascularization (10, 11).

In addition to these positive signals, the extracellular matrix may also provide negative signals to regulate endothelial cell growth. TSP1 (reviewed in Refs. 12 and 13) is one of several matricellular components that, under defined conditions, can inhibit endothelial cell adhesion (14, 15), motility (16), and growth (16–19). TSP1 specifically inhibits endothelial cell adhesion on a fibronectin matrix (14). However, TSP1 can also act as a positive stimulator of endothelial cell adhesion and motility (16, 20), and both positive and negative effects of TSP1 have been reported on angiogenesis *in vivo* (reviewed in Refs. 21–23). Conflicting signals may therefore arise from the interaction of endothelial cells with TSP1, and further work is needed to define the integration and regulation of these responses. In our experience, however, expression of TSP1 in human breast carcinoma cells suppresses their tumorigenic and angiogenic activity in mouse xenografts (24, 25).

Three domains of TSP1 are implicated to date in the inhibitory activities of TSP1 on endothelial cell growth and motility. The amino-terminal domain of TSP1 mimics the inhibitory activity of intact TSP1 on focal adhesion contacts, and this activity is suppressed by an antibody to this domain (26). The recombinant amino-terminal domain of TSP1 also inhibits proliferation and motility of endothelial cells in response to FGF-2 (27). The TSP1 procollagen domain peptide NGVQYRN inhibits motility of endothelial cells to FGF-2 *in vitro* and angiogenesis *in vivo* (28), but its mechanism of action is not known. The type I repeats of TSP1 contain additional inhibitory peptide sequences (27, 28). Peptides from the type I repeats compete with FGF-2 for binding to endothelial cells and inhibit both proliferative and motility responses to this growth factor (27). Because the type I repeat peptides also compete for binding of FGF-2 to heparin or to intact endothelial cells, we proposed that these peptides inhibit endothelial cell responses to FGF-2 by competing with the growth factor for binding to the heparan sulfate proteoglycans that are required for presenting FGF-2 to its high affinity tyrosine kinase receptor (27). On the basis of our recent identification of a TGF-β-activating sequence in this same peptide (29), a second possible mechanism for the observed growth inhibition by TSP1 is by activation of latent TGF-β produced by the endothelial cells (30).

We also observed that endothelial cells lose their normal morphology when treated with peptides from the type I repeats of TSP1 and that cell numbers decreased after incubation with the peptides.[4] Although the peptides also inhibited the growth of a human breast carcinoma cell line, we did not observe a decrease in cell number. This suggested that the peptides may either have a specific cytotoxic activity toward endothelial cells or trigger programmed death of these cells. We have further examined the effects of TSP1 and the peptides on endothelial and breast carcinoma cells, and we report here that TSP1 and the thrombospondin peptides specifically induce apoptosis

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[3] The abbreviations used are: FGF-2, fibroblast growth factor 2; BAE, bovine aortic endothelial; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TGF-β, transforming growth factor β1; TSP1, thrombospondin 1; BrdUrd, bromodeoxyuridine.

[4] N. Guo, H. C. Krutzsch, J. K. Inman, and D. D. Roberts. Antiproliferative and antitumor activities of D-reverse peptide mimetics derived from the second type-1 repeat of thrombospondin 1, submitted for publication.

in endothelial cells and that this activity is independent of their ability to activate latent TGF-β.

MATERIALS AND METHODS

TSP1 was purified from thrombin-stimulated human platelets as described previously (31). Fibronectin was purified from human plasma (NIH Blood Bank) as described (32). Recombinant human TGF-β1 was obtained from R&D Systems, Inc. Synthetic peptides from the type I repeats of human TSP1 were prepared and characterized as described previously (33, 34). Analogues of the TSP1 sequence (KRAKAAGGWSHWSPWSSC, KRFKQDGGASHASPASSC) were prepared with appropriate Ala substitutions to eliminate the essential Phe residue for TGF-β activation or the Trp residues required for heparin binding and contain a carboxyl-terminal Cys residue to allow conjugation to polysucrose. Structures of the peptides used are summarized in Table 1. Peptides with Ala substitutions for Phe were unable to activate a mixture of latent TGF-β1 and TGF-β2 in BAE cell conditioned medium as assessed by NRK fibroblast colony formation in soft agar. Conjugation of the peptides to polysucrose was performed as described previously.[4] In all cases, the peptides were used as polysucrose conjugates, which lack the adhesive activity of the free peptides but retain their other biological activities to regulate cell proliferation.[4]

Cell Culture. BAE cells were kindly provided by Dr. E. Gallin (Armed Forces Radiobiology Research Institute, Bethesda, MD) and were used at passages 4–10. BAE cells were maintained at 37°C in 5% CO_2 in DMEM (low glucose) containing 10% FCS, 4 mM glutamine, 25 μg/ml ascorbic acid, and 500 units/ml each of penicillin G, potassium, and streptomycin sulfate. Media components were obtained from Biofluids Inc. (Rockville, MD). MDA-MB-435S and MCF7 breast carcinoma cells (American Type Culture Collection) were grown in RPMI 1640 containing 10% FCS. Okadaic acid, TPA, fumonisin B1, herbimycin A, and sodium vanadate were purchased from Sigma.

TGF-β Assays. NRK fibroblast bioassays for TGF-β activity were conducted as described previously (29, 35). Serum-free conditioned medium (35) prepared from BAE cells at 70% confluence was used as a source of latent TGF-β for detecting activation by synthetic peptides in the NRK colony-forming assay. NRK colonies in soft agar were quantified microscopically. TGF-β1 was also quantified using an immunoassay specific for this isoform (Genzyme Corp.). BAE cells secrete latent TGF-β1 and TGF-β2 (36), and MDA-MB-435S cells produce TGF-β1, TGF-β2, and TGF-β3 (37). Using the TGF-β1-specific ELISA, 24-h conditioned media from BAE, MCF7, and MDA-MB-435S cells contained 2.05, 2.53, and 1.68 ng/ml acid-activatable TGF-β1. More than 90% of total TGF-β activity was latent in media from each cell line as assessed by the NRK bioassay.

Endothelial Cell Proliferation. Cell proliferation was measured using the Cell-Titer colorimetric assay (Promega) as described previously (27). In brief, cells were trypsinized and suspended in complete medium (BAE cells in DMEM; MDA-MB-435S cells in RPMI 1640) containing 10% FCS at 1–1.5 $\times$ 10^5 cells/ml. Inhibitors were added to a 96-well plate (Costar Corp.) in 50 μl of medium without FCS followed by 50 μl of the endothelial cell suspension. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 72 h. For determining the effect of okadaic acid, phorbol, herbimycin, fumonisin B1, or TPA on proliferation, the reagents were prepared in medium and added into wells together with the protein or peptide inhibitors.

DNA Fragmentation ELISA. DNA fragmentation was quantified by an ELISA assay (Boehringer Mannheim, Indianapolis, IN) using BrdUrd-labeled cells. A 10-ml suspension of target cells (BAE cells or MDA-MB-435S or MCF7 cells, 10 ml at 2 $\times$ 10^5 cells/ml) was plated and incubated overnight at 37°C in a humidified atmosphere with 10 μM BrdUrd added to 10 ml of low-glucose DMEM with 10% FCS. After labeling, the cells were trypsinized and centrifuged at 250 $\times$ g for 3 min and resuspended in culture medium. The cell concentration was adjusted to 1 $\times$ 10^5 cells/ml, and 100 μl/well were transferred to replicate wells of a microtiter plate containing either medium with peptides or culture medium only (100 μl/well) to yield a final volume of 200 μl/well. The cells were incubated for 24–72 h as indicated at 37°C in a humidified atmosphere with 5% CO_2. For determining the effect of inhibitors on confluent endothelial cells, cells were cultured for 24–48 h at 37°C until the cells reached confluence, and inhibitors were added into the wells and incubated as described above.

After incubation, the cells in the plate were lysed by adding 20 μl of washing buffer (10×) for 30 min at room temperature. The microtiter plate was centrifuged at 250 $\times$ g for 10 min, and 100 μl of supernatant were transferred into the wells of a microtiter plate precoated with anti-DNA antibody. The samples were incubated for 90 min at room temperature. After washing, the samples were denatured and fixed by microwave irradiation of the plate for 5 min. After cooling the plate for 10 min at −20°C, anti-BrdUrd peroxidase conjugate solution was added and incubated for an additional 90 min at room temperature. After washing, immune-complexed anti-BrdUrd peroxidase was detected by 3,3′,5,5′-tetramethylbenzidine substrate. After incubation for 10–20 min at room temperature in the dark, absorbance was detected by monitoring at 450 nm.

Gel Analysis of DNA Fragmentation. Cells (5 $\times$ 10^5/well in 1.5 ml) were cultured on 6-well Nunc tissue culture plates in 10% FCS complete medium for 24 h. The medium was replaced with medium containing 5% FCS and the inhibitors to be tested. After incubating for 24 h at 37°C, the cells were removed by trypsinization and collected by centrifugation at 1,000 rpm for 3 min in complete medium. Lysis buffer [0.5 ml; containing 5 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 100 mM 2-mercaptoethanol, and 0.5 mg/ml proteinase K] was added to the cell pellet and incubated at 37°C for 30 min (38). The lysate was vortexed for 15 s and precipitated with an equal volume of isopropanol at −70°C for 1 h. The samples were centrifuged for 30 min at 12,000 $\times$ g at 4°C, and the DNA pellets were washed in 70% ethanol at room temperature. After drying in a Speedvac concentrator for 15 min, the samples were dissolved in 25 μl of Tris-EDTA buffer containing 0.6 mg/ml RNase A and incubated overnight at 37°C. The samples were reextracted, washed, and dried as described above. The pellets were dissolved in 30 μl of Tris-EDTA buffer, and the DNA was subjected to electrophoresis on a horizontal 2% agarose gel in Tris-borate EDTA buffer. The DNA was stained with SYBR green solution (Molecular Probes, Inc., Eugene, OR) diluted 1:5,000 in running buffer.

RESULTS

We have previously shown that peptides derived from the second type I repeat of TSP1 inhibit proliferation of endothelial cells and a breast carcinoma cell line (27).[4] Some of these peptides also promote cell adhesion (34).[4] Because these two activities may elicit opposing signals in cells,[4] polysucrose conjugates of the peptides, which do not promote cell adhesion, were used instead of free peptides in the current studies. The peptide conjugates arrested growth of both endothelial and breast carcinoma cells, but endothelial cell numbers also decreased after this treatment. The decrease in endothelial cell number was preceded by morphological changes in the treated endothelial cells that are characteristic of programmed cell death (Fig. 1*c*), including membrane blebbing, nuclear condensation, and loss of adhe-

Table 1 *Structures of TSP1 peptides and mimetics*

Peptide	Origin	Sequence[a]
407	TSP1 type 1 (residues 429–447)	KRFKQDGGWSHWSPWSSC
389	peptide 407 ($Trp^3 \rightarrow Ala^3$)	KRFKQDGGASHASPASSC
450	peptide 407 (Phe, GlnAsp $\rightarrow$ Ala^3)	acKRAKAAGGWSHWSPWSSCam
416	peptide 407 (retro-inverso)	all D-acCSSWPSWHSWGGDQKFRKam
500	TSP1 procollagen (residues 321–327)	NGVQYRNC
493	TSP1 residues 436–444 (retro-inverso)	all D-tpAAWPSWHSWGGam
521	TSP1 residues 429–446 (retro-inverso)	all D-tpSSWPSWHSWGGDQKFRKam

[a] Sequences are depicted using single-letter codes and are L-amino acids except where indicated; tp, thiopropionyl; am, amide; ac, acetyl.

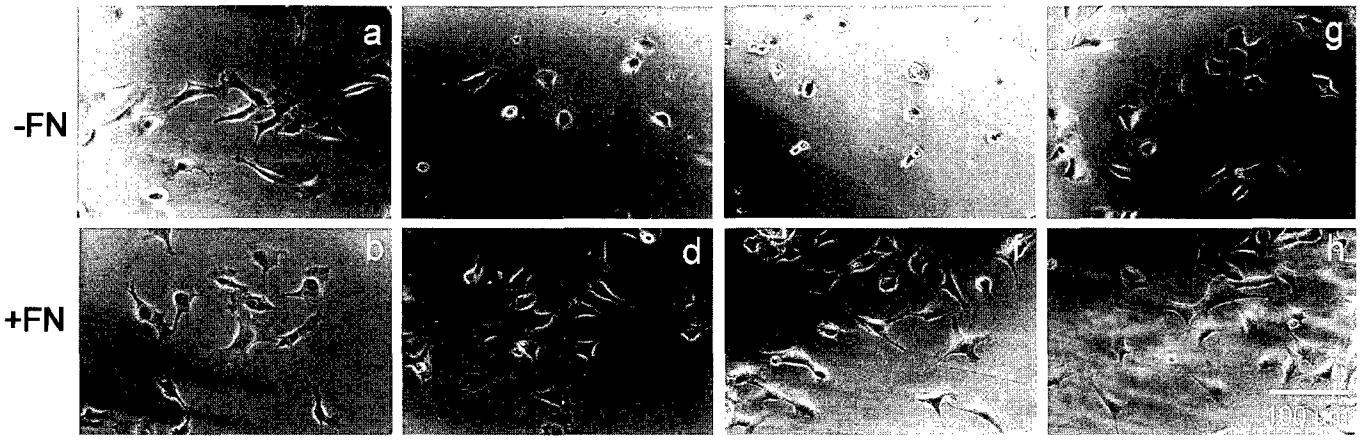

Fig. 1. Effect of TSP1 type I repeat peptides on morphology of BAE cells. Cells were photographed 22 h after plating on tissue culture plastic (*a, c, e,* and *g*) or on plastic coated with 10 μg/ml human plasma fibronectin (*b, d, f,* and *h*). Cells in *c–h* were treated with polysucrose conjugates containing 4 μM of the TSP1 type 1 repeat peptide KRFKQDGGWSHWSPWSSC (*c* and *d*), an analogue without the TGF-β-activating sequence acKRAKAAGGWSHWSPWSSCam (*e* and *f*), or the TSP1 procollagen domain peptide NGVQYRNC (*g* and *h*).

sion. The morphological changes were specifically induced by the type I repeat peptides because a control conjugate containing an antiangiogenic peptide from the procollagen domain of TSP1 was inactive (Fig. 1*g*).

BAE cells secrete latent TGF-β1 and TGF-β2 (36), and the TSP1 sequence KRFK activates latent TGF-β (29). Conditioned medium from the BAE cells used for this assay contained 0.01 ng/ml TGF-β1 immunoreactivity and 2.05 ng/ml acid-activatable TGF-β1. Because similar morphology changes were induced by a modified TSP1 sequence in which the TGF-β-activating sequence KRFK was altered to the inactive sequence KRAK (Fig. 1*e*), the effect of the peptide did not require activation of latent TGF-β produced by the endothelial cells.

Induction of DNA Fragmentation. Analysis of low molecular weight DNA extracted from endothelial cells treated with the active TSP1 peptides demonstrated a characteristic ladder pattern resulting from internucleosomal cleavage of the genomic DNA (Fig. 2). Polysucrose conjugates containing 0.4 μM of either the native TSP1 sequence KRFKQDGGWSHWSPWSSC (*Lane b*) or the modified sequence KRAKAAGGWSHWSPWSSC (*Lane e*), which lacks the TGF-β-activating sequence, equally stimulated DNA fragmentation. The basic residues and the WSXW motif were both required for optimal activity of these peptide conjugates, based on the weak activities of conjugates containing KRFKQDGGASHASPASSC (*Lane a*) or GGWSHWSPWSSC (*Lane d*), which lack either the Trp residues or the basic motif. The appearance of cleaved DNA fragments was specific for the active type I repeat peptides because a polysucrose conjugate containing the TSP1 procollagen peptide NGVQYRNC was inactive (*Lane f*). Two conjugated retro-inverso mimetics of the type I sequence were also active (*Lanes c* and *g*). Exposure of the cells to 1 μg/ml TSP1 did not result in detectable DNA fragmentation by this method (*Lane h*).

Apoptosis Is Blocked on a Fibronectin Matrix. Because loss of matrix adhesion is a known inducer of apoptosis in endothelial cells (8, 9), an antiadhesive activity was considered as a mechanism for the activity of the peptides. Precoating the tissue culture plastic with fibronectin did not alter the morphology of untreated cells (Fig. 1, *a* and *b*) but prevented the morphology changes induced by the TSP1 peptides (Fig. 1, *d* and *f*). The antiproliferative activities of the native TSP1 sequence (KRFKQDGGWSHWSPWSSC-polysucrose, 407f) and an analogue without the TGF-β-activating sequence RFK (KRAKAAGGWSHWSPWSSC-polysucrose, 450f) were also decreased by growth of the endothelial cells on a fibronectin matrix (Fig. 3). In contrast, the antiproliferative activities of intact TSP1 or TGF-β were not significantly decreased by attachment of the endothelial cells on fibronectin (Fig. 3).

Fibronectin also inhibited the appearance of the DNA ladder in endothelial cells treated with a TSP1 peptide analogue from the type 1 repeat (Fig. 4). DNA fragmentation induced by the peptide KRFKQDGGWSHWSPWSSC conjugate was reduced by 84% in cells attached on wells coated with 10 μg/ml fibronectin. A conjugate containing the TSP1 procollagen peptide NGVQYRNC was used as a negative control in this experiment and did not significantly induce DNA fragmentation. Similar reductions in DNA fragmentation were observed for the other active TSP1 peptide analogues when cells were attached on fibronectin (data not shown).

Quantitative Analysis of DNA Fragmentation. An ELISA assay for detecting DNA fragmentation was used to quantify the activity of

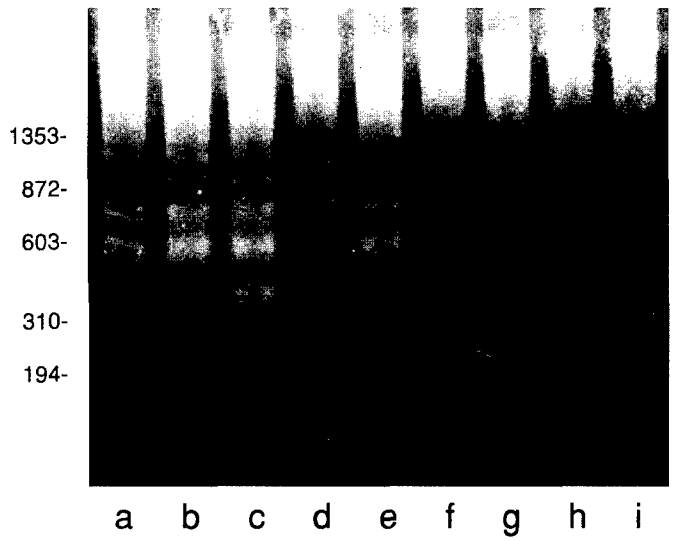

Fig. 2. Type I repeat peptide analogues from TSP1 induce DNA fragmentation in aortic endothelial cells. BAE cells (5×10^5) were treated with 0.4 μM of the indicated TSP1 peptide conjugates or 25 μg/ml of intact TSP1 for 24 h. Low molecular weight DNA was extracted from the cells and analyzed by electrophoresis on a 2% agarose gel. DNA fragments were visualized by staining with SYBR green. Cells were treated with polysucrose conjugates of the following peptides: KRFKQDGGASHASPASSC, *Lane a*; KRFKQDGGWSHWSPWSSC, *Lane b*; retro-inverso amKRFKQDGGWSHWSPWSS-Cac, *Lane c*; GGWSHWSPWAAC, *Lane d*; KRAKAAGGWSHWSPWSSC, *Lane e*; NGVQYRNC, *Lane f*; retro-inverso amKRFKQDGGWSHWSPWSS-thioproprionyl, *Lane g*; TSP1, *Lane h*; and control, *Lane i*. *Left margin*, migration of DNA size markers.

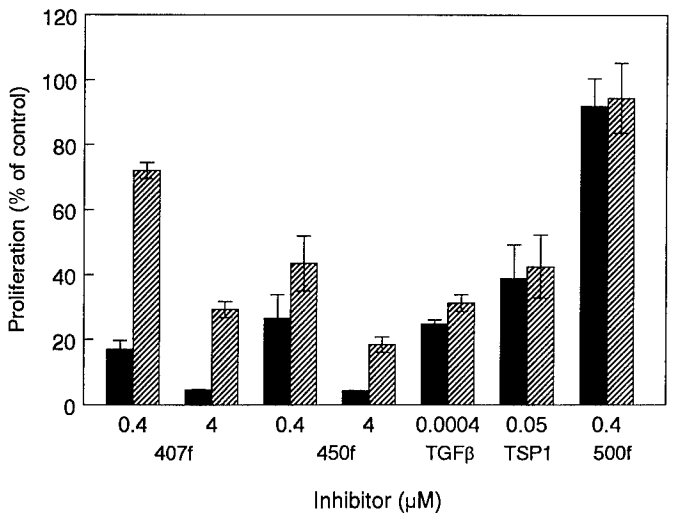

Fig. 3. Attachment of endothelial cells on fibronectin partially reverses the inhibition of proliferation by type 1 repeat peptides but not by TSP1 or TGF-β. Proliferation of BAE cells was determined on untreated tissue culture plastic (■) or on plastic coated with 10 μg/ml fibronectin (▨) in DMEM medium containing 1% FBS and the indicated concentrations of the TSP1 peptide KRFKQDGGWSHWSPWSSC (*407f*), an analogue without the TGF-β-activating sequence acKRAKAAGGWSHWSPWSSCam (*450f*), TGF-β1 (*TGF-β*), TSP1, or the TSP1 procollagen domain peptide NGVQYRNC (*500f*). The cell number was quantified after 72 h using the Cell-Titer assay and is presented as a percentage of that determined in the same medium without additions, mean ± SD, n = 3.

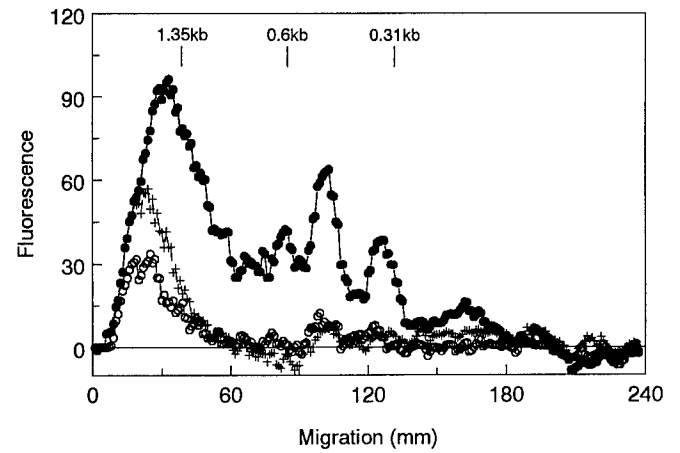

Fig. 4. Fibronectin inhibits TSP1 peptide-induced DNA fragmentation. DNA fragmentation was determined as described in the legend to Fig. 2. Band intensity was determined by image analysis and is plotted with background subtraction using control cells as a reference for endothelial cells treated with a conjugate of peptide KRFKQDGGWSHWSPWSSC (●), cells treated with the same peptide in a well coated with 10 μg/ml fibronectin (+), or cells treated with a conjugate of the TSP1 procollagen peptide NGVQYRNC (○). The migration of DNA size standards is as indicated.

the peptides. On the basis of this sensitive and quantitative assay for DNA fragmentation, both TSP1 and the type I repeat peptides induced significant DNA fragmentation in endothelial cells (Fig. 5*A*). The activity of TSP1 was weaker than that of the synthetic peptide conjugates to elicit DNA fragmentation but was consistently observed in several independent experiments. The TSP1 procollagen domain peptide, however, was inactive. Treatment of BAE cells with TGF-β induced DNA fragmentation to a similar extent as TSP1. The stimulation of DNA fragmentation by TSP1 and the peptide conjugates was specific because no DNA fragmentation was induced in MDA-MB-435S breast carcinoma cells by the peptide conjugates (Fig. 5*B*). DNA fragmentation was induced in MDA-MB-435S cells by the topoisomerase I inhibitor camptothecin, indicating that these cells can initiate programmed cell death. Because the same peptides inhibited the growth of MDA-MB-435S cells,[4] the induction of apoptosis can be independent of the antiproliferative effects of the TSP1 peptides. A second breast carcinoma cell line, MCF7, showed DNA fragmentation in response to the peptide 407 conjugate but not to intact TSP1 (Fig. 5*B*). The magnitude of the peptide response was similar to the DNA fragmentation induced in the same cells by camptothecin.

On the basis of the protective activity of fibronectin observed in Figs. 1 and 4, the effect of endothelial cell adhesion on the induction of DNA fragmentation was further examined using the quantitative DNA fragment ELISA. The adhesion of endothelial cells on a fibronectin matrix inhibited DNA fragmentation induced by TSP1 (Fig. 6*A*) or by a TSP1 peptide analogue from the type I repeats (Fig. 6*B*). Consistent with the report that vanadate suppresses the induction of endothelial cell death induced by removal of extracellular matrix signals (8), the addition of 50 μM vanadate decreased the fragmentation induced by the active TSP1 peptide KRFKQDGGWSHWSPWSSC (407f) or by the analogue lacking the TGF-β-activating sequence (450f; Fig. 7). The serine/threonine phosphatase inhibitor okadaic acid, at 5 nM, also inhibited DNA fragmentation induced by these peptides, whereas the ceramide

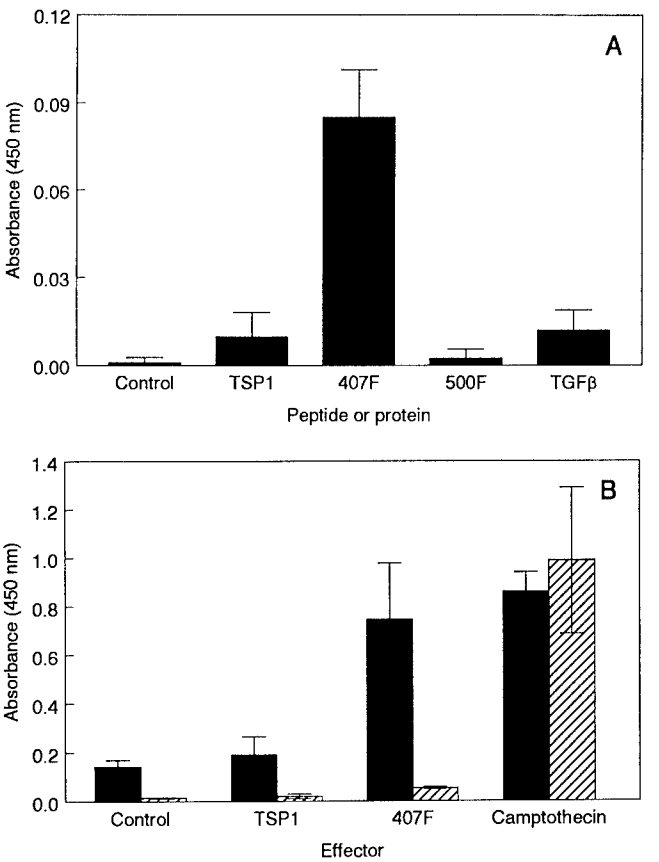

Fig. 5. Detection of TSP1- and peptide-induced apoptosis in BAE and human breast carcinoma cells by a DNA fragment ELISA. DNA fragmentation in BAE cells (*A*), MCF7 breast carcinoma cells (*B*, ■), or MDA-MB-435S breast carcinoma cells (*B*, ▨) was quantified by an ELISA assay using BrdUrd-labeled cells. Target cells (10 ml; 2×10^5 cells/ml) were labeled overnight using 10 μM BrdUrd. After labeling, a cell suspension containing 1×10^5 cells/ml was transferred to replicate wells of a microtiter plate (100 μl/well) containing 100 μl of culture medium containing inhibitors (25 μg/ml TSP1, 4 μM KRFKQDGGWSHWSPWSSC (*407F*), NGVQYRNC (*500F*), 400 ng/ml camptothecin, or 10 ng/ml TGF-β) or medium only (*Control*). After incubation for 24 h at 37°C, the cells in the wells were lysed and centrifuged. Released DNA fragments in 100 μl of the supernatant were quantified using a sandwich ELISA using anti-DNA capture antibody and anti-BrdUrd peroxidase conjugate for detection. After washing, immune-complexed anti-BrdUrd peroxidase was detected using 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance was measured at 450 nm and is presented as mean ± SD, n = 3.

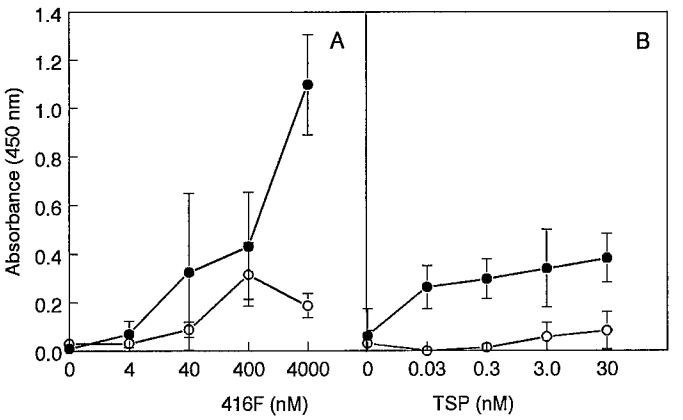

Fig. 6. Fibronectin protects endothelial cells from DNA fragmentation induced by TSP1 and a peptide from the type I repeat. DNA fragmentation in BrdUrd-labeled endothelial cells was detected using an ELISA assay. BAE cells were grown in DMEM with 5% FCS in tissue culture wells (●) or wells coated with 10 μg/ml fibronectin (○) with the indicated concentrations of a TSP1 peptide analogue (retro-inverso amKRFKQDGGWSHWSPWSSCac, *A*) or human platelet TSP1 (*B*). TSP1 concentration is expressed on a subunit molar basis. Colorimetric detection of DNA fragment release was determined in triplicate and is presented as mean ± SD.

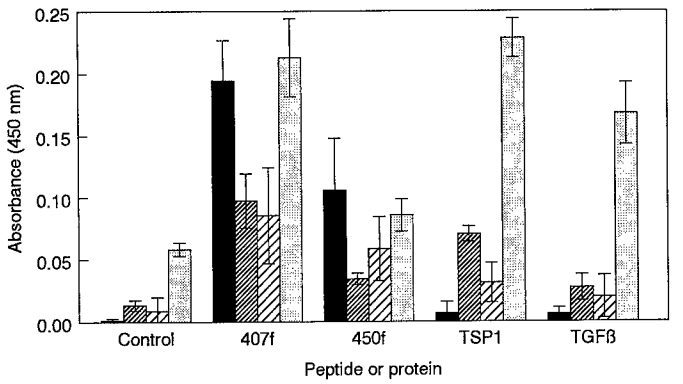

Fig. 7. Modulation of TSP1- and peptide-induced DNA fragmentation in BAE cells. Cells were plated in DMEM containing 5% FCS (■) or the same medium containing 50 μM vanadate (*narrow stripes*), 5 nM okadaic acid (*wide stripes*), or 25 nM fumonisin B1 (▨). The indicated peptides or proteins (4 μM 407f or 450f, 50 nM TSP1, and 0.4 nM TGF-β) were added and incubated for 36 h at 37°C in 5% CO_2. DNA fragmentation was detected by ELISA, and the results are presented as mean ± SD, $n = 3$.

synthase inhibitor fumonisin B1 did not. In contrast, the tyrosine and serine/threonine phosphatase inhibitors unexpectedly increased DNA fragmentation induced by intact TSP1, as did fumonisin B1. Alterations in the DNA fragmentation response to TGF-β in the presence of these inhibitors paralleled those of TSP1, suggesting a common mechanism of action or that TGF-β bound to the TSP1 is responsible for the observed response. The TGF-β concentration in the TSP1 was quantified using an ELISA assay specific for TGF-β1 (Genzyme Corp.). No TGF-β1 immunoreactivity was detected without acid treatment of the TSP1 used in these experiments. After acid treatment, 0.07 ng of TGF-β1 was detected per microgram of TSP1. Assuming that all of this TGF-β1 is active, it would account for only 17% of the observed TSP1 response.

A role for adhesion in regulating the sensitivity of endothelial cells to the TSP1 peptides was also suggested by its dependence on cell density (Fig. 8). Maximal sensitivity to induction of DNA fragmentation by the TSP1 peptide KRFKQDGGWSHWSPWSSC was observed using subconfluent cells, whereas the induction of DNA fragmentation was suppressed when confluent endothelial cells were used. The addition of the phosphatase inhibitor vanadate to the subconfluent cells produced a similar suppression of the DNA fragmentation response to the peptide (Fig. 8).

Phosphorylation Differentially Modulates Antiproliferative Responses to Peptides and Thrombospondin. On the basis of the observation that phosphatase inhibition differentially affected apoptotic responses to TSP1 and the peptides, we further examined the mechanism of endothelial growth inhibition by the TSP1 type I repeat peptides. Sodium vanadate significantly inhibited the antiproliferative activity of TSP1 and TGF-β but did not inhibit the antiproliferative activity of the TSP1 peptides (Table 2). Blocking of the TSP1 and TGF-β activities by vanadate was specific in that the serine/threonine phosphatase inhibitor okadaic acid had no effect at a concentration sufficient to inhibit protein phosphatase 2A (39). However, at concentrations sufficient to inhibit protein phosphatase 1 (25 nM), okadaic acid alone inhibited endothelial growth and strongly induced DNA fragmentation (results not shown).

Although the activity of the phosphatase inhibitor vanadate to antagonize the antiproliferative effect of TSP1 suggests that hyperphosphorylation prevents the antiproliferative activity of TSP1, a tyrosine kinase may also mediate the growth-suppressive activity of TSP1 and the peptides. The tyrosine kinase inhibitor herbimycin A, used at concentrations below those that directly blocked endothelial

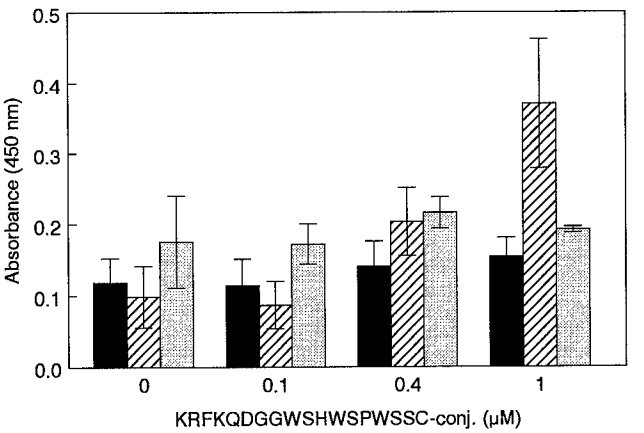

Fig. 8. Endothelial cell density and vanadate modulate DNA fragmentation induced by a peptide from the type I repeat of TSP1. Subconfluent BAE cells (1×10^4 cells/well) with (▨) or without (▨) 100 μM vanadate or confluent BAE cells (1×10^4 cells/well and precultured for 48 h, ■) were labeled with BrdUrd and incubated for 24 h in DMEM containing 5% FCS and the indicated concentrations of the TSP1 type 1 repeat peptide conjugate. DNA fragment release was quantified by ELISA and is presented as mean ± SD, $n = 3$.

Table 2 *Effect of signal transduction modulators on inhibition of endothelial cell proliferation by TSP1, TGF-β, and TSP1 peptides*

Inhibition of BAE cell proliferation by TSP1, TGF-β, or the indicated thrombospondin peptide analogues were determined in the presence of inhibits of tyrosine kinase (herbimycin), ceramide synthase (fumonisin), phosphatases (vanadate or okadaic acid), or a stimulator of protein kinase C (TPA). Net inhibition of proliferation, expressed as mean ± SD for triplicate wells, was determined relative to control cells treated with the same inhibitors or activators in the absence of the test proteins or peptides.

	% Inhibition of proliferation				
	450F (0.4 μM)	500F (0.4 μM)	407F (0.4 μM)	TSP1 (150 nM)	TGFβ (0.4 nM)
Control	49 ± 4	−4 ± 4	97 ± 1	70 ± 4	71 ± 2
Herbimycin (1 μM)	0 ± 5	0 ± 9	50 ± 7	−14 ± 12	−5 ± 12
Fumonisin (15 μM)	24 ± 3	6 ± 4	97 ± 1	74 ± 2	84 ± 3
Control	59 ± 4	−5 ± 19	74 ± 5	61 ± 3	72 ± 1
Vanadate (20 μM)	61 ± 3	−3 ± 8	83 ± 5	−8 ± 12	26 ± 7
Okadaic acid (5 nM)	77 ± 5	17 ± 4	96 ± 1	65 ± 4	80 ± 1
Control		1 ± 5	43 ± 2	52 ± 1	
TPA (50 nM)		1 ± 2	64 ± 6	48 ± 1	
Phorbol (50 nM)		2 ± 6	77 ± 2	57 ± 2	

proliferation, strongly suppressed the antiproliferative activities of the TSP1 peptides and completely blocked the antiproliferative activities of TSP1 and TGF-β (Table 2). Because herbimycin also blocked the antiproliferative activity of the TSP1 peptide analogue 450, which lacks a latent TGF-β-activating sequence, herbimycin can prevent activity of the TSP1 peptides independently of blocking TGF-β-mediated signaling. In contrast, fumonisin B1, an inhibitor of ceramide synthase and ceramide-mediated apoptosis (40), had no effect on the activity of the peptides at 15 μM (K_i = 0.2 μM for ceramide synthase; Ref. 40). At higher doses, fumonisin B1 also inhibited endothelial cell proliferation and directly induced DNA fragmentation (results not shown).

The protein kinase C stimulator TPA, which blocks ionizing radiation-induced ceramide generation and apoptosis of BAE cells (41, 42), stimulated proliferation of the cells but had no effect on the antiproliferative activities of the TSP1 peptides or TSP1 (Table 2). The inactive analogue 4α-phorbol did not stimulate proliferation, verifying the specificity of the proliferative response to TPA. TPA also had no effect on the generation of DNA fragmentation induced by the TSP1 peptides as assessed by the DNA ladder assay (results not shown).

DISCUSSION

Previous studies have demonstrated the positive effects of extracellular matrix components on endothelial cell survival (8, 9, 11). Recently, however, TSP1 and several other matricellular components have been found to negatively modulate cell adhesion (12, 43). Because adhesion provides signals essential for survival of nontransformed cells, these observations suggested that TSP1 may also regulate cell survival. The present results demonstrate a negative effect of TSP1 on endothelial cell survival. The ability of TSP1 or the TSP1 peptide analogues to inhibit growth and induce apoptosis, however, is dependent on other external signals. Confluent quiescent cells were resistant to the induction of cell death, as were subconfluent cells attached to a pure fibronectin matrix or cells treated with vanadate. As was demonstrated for the interaction of cells with fibronectin (4), the signals resulting from the interaction of endothelial cells with TSP1 may be complex and involve multiple signal transduction pathways. TSP1 and the TSP1 peptides elicit changes in both endothelial cell proliferation and survival. On the basis of their differential sensitivities to fibronectin matrix signals and agents that modulate several signal transduction pathways, these responses probably involve discreet signaling pathways.

Several results indicate that induction of apoptosis is independent of the growth-inhibitory activities of TSP1 and the TSP1 type I repeat peptides. Proliferation of breast carcinoma and endothelial cells are both inhibited by TSP1 and the peptides,[4] but only the latter cells exhibited an apoptosis response. Differential sensitivity of the endothelial cell apoptosis and proliferative responses to vanadate inhibition for the TSP1 peptides also suggest that distinct mechanisms may mediate growth inhibition and apoptosis. Likewise, the proliferative and survival responses to intact TSP1 differ in that fibronectin reversed the apoptotic response but did not reverse the antiproliferative activity of TSP1.

The parallel proliferative and survival responses of TSP1 and TGF-β-treated endothelial cells to many of the inhibitors tested suggest that TGF-β may mediate the activity of intact TSP1, although it is not required for activity of the TSP1 peptides. Part of the observed response to TSP1 could result from active TGF-β contaminating the platelet TSP1, but the measured concentration of TGF-β was insufficient to account for most of the activity observed. The TSP1 may also activate latent TGF-β1 produced by the BAE cells (30). As was observed with the peptides, however, sensitivities to signal transduction inhibitors differ for TSP1-mediated growth inhibition and induction of DNA fragmentation. Vanadate completely reversed the antiproliferative activity of TSP1 but augmented DNA fragmentation. Likewise, fumonisin B1 had no effect on the antiproliferative activity of TSP1 but also augmented DNA fragmentation. This pattern parallels previous reports that apoptotic and growth-inhibitory responses to TGF-β may also involve distinct signaling pathways (38).

Peptides from the type I repeats of TSP1 elicit a strong apoptotic response in endothelial cells. To date, no other region of TSP1 has been found to induce apoptosis, and the present data exclude this as a mechanism for the antiangiogenic activity of the procollagen domain peptide (28). Mutagenesis of the type I repeat sequences will be required to confirm the role of the type 1 repeats in the activity of the intact protein and to determine whether other regions of TSP1 participate in the cell death response to intact TSP1.

The mechanism of action of the TSP1 peptides is clearly not from direct cytotoxicity, based on the resistance of endothelial cells plated on fibronectin or at confluence to apoptosis in the presence of active concentrations of the peptides. The peptides may act outside of the cell to block FGF-2 presentation to and activation of its tyrosine kinase receptor (27). This hypothesis is consistent with protection by vanadate from peptide-mediated apoptosis. However, some other results question this hypothesis. Partial reversal of the peptide antiproliferative activities by herbimycin and the lack of vanadate sensitivity are not consistent with their acting by antagonizing a tyrosine kinase-dependent receptor. Furthermore, FGF-2 is known to suppress ceramide-mediated apoptosis of BAE cells, and this activity is mediated by protein kinase C (42). Because TPA stimulation of PKC protects BAE cells from ceramide-mediated apoptosis but did not protect our BAE cells from an inhibition of growth by the TSP1 peptides, the antiproliferative activity of the peptides cannot arise exclusively from inhibition of an essential FGF-2 survival signal. Although ceramide has recently been shown to mediate apoptosis of many cell types in response to various stimuli (reviewed in Ref. 44) and participates in radiation-induced apoptosis of BAE cells (41, 42), the lack of effect of fumonisin B1 and TPA on the activities of the peptides suggests that the pathway for inducing cell death by the TSP1 peptides is distinct from that of ionizing radiation and does not require ceramide generation. On the basis of the apparent synergism of fumonisin B1 with TSP1 to induce DNA fragmentation, however, the apoptotic response to the intact protein may be regulated by ceramide generation.

TSP1 inhibits focal adhesion contacts in endothelial cells attached on fibronectin (15). This mechanism could participate in the activity of TSP1 but not that of the peptides because the amino-terminal domain of TSP1 is responsible for the former activity. Treatment with the peptide conjugates, however, also results in the loss of endothelial cell adhesion. It remains to be determined whether this loss of adhesion causes programmed cell death or is an indirect effect of other signals induced in the cells by the peptides.

Attachment of the endothelial cells to fibronectin or being at confluence generates a signal that reverses the apoptotic and antiproliferative responses to the peptides. Because fibronectin binding to the integrin $\alpha5\beta1$ promotes endothelial cell survival (8), this signal may involve activation of focal adhesion kinase or other adhesion-dependent tyrosine kinases. Fibronectin or antibody engagement of $\beta1$ or $\beta3$ integrins on endothelial cells results in tyrosine kinase-dependent phosphorylation of focal adhesion kinase and a 70-kDa protein (45). Vanadate can replace the fibronectin signal to prevent endothelial cell death (8) and presumably maintains the targets of these kinases in a phosphorylated state by inhibiting the corresponding phosphatases. This model is consistent with the ability of vanadate to suppress DNA fragmentation induced by the TSP1 peptide KRFKQDGGWSHWSPWSSC and to reverse growth inhibition by intact TSP1. However, it

does not account for the ability of vanadate to stimulate apoptosis induced by intact TSP1 or the ability of herbimycin A to prevent growth inhibition by TSP1 or the peptides. The latter result was also unexpected because herbimycin is reported to inhibit angiogenesis (46), most integrin signaling (4, 45), and apoptotic responses in several cell types (47). The data can be rationalized by proposing that the peptides elicit a second inhibitory tyrosine kinase pathway that is sensitive to herbimycin.

The role of programmed cell death in the biological activities of TSP1 *in vivo* remains to be examined. TSP1 overexpression in MDA-MB-435S breast carcinoma cells reduced tumor growth *in vivo* but had no effect on the growth of these cells or the formation of colonies in soft agar (24). These observations are consistent with the inability of TSP1 to induce apoptosis of MDA-MB-435S cells. The resistance of MDA-MB-435S breast carcinoma cells to induction of apoptosis by the TSP1 peptides may result from mutation of *p53* in this cell line (48), whereas the MCF7 cells have wild-type *p53* and are sensitive to the induction of apoptosis. Normal p53 function may therefore be required for the apoptotic response to TSP1 peptides.

Reduction of angiogenesis in tumors formed by TSP1-transfected MDA-MB-435S cells (24) could result from the induction of apoptosis in endothelial cells during vascularization of the tumor. A similar mechanism has been proposed for the antitumor activity of antibodies to the $\alpha v\beta 3$ integrin, which induce apoptosis in developing tumor blood vessels (10, 11). Thus, extracellular matrix signals may be absent in newly formed tumor blood vessels and sensitize this endothelium to the effects of TSP1 secreted by the transfected MDA-MB-435S cells. Although TSP1 is a ligand for $\alpha v\beta 3$ (20), the active TSP1 peptides do not contain the Arg-Gly-Asp sequence recognized by the $\alpha v\beta 3$ integrin.

The TSP1 peptides are potent inducers of DNA fragmentation in BAE cells *in vitro*. This activity may account for the differential effects of these peptides on endothelial and breast carcinoma cell proliferation *in vitro*. We recently found that stable analogues of the TSP1 peptides inhibit tumor growth *in vivo* in MDA-MB-435S xenografts in nude mice.[4] The present data suggest that the selective induction of apoptosis of tumor endothelium may explain the activity of the peptides *in vivo*. The resistance of confluent endothelial cells to the induction of apoptosis by the peptides *in vitro* is consistent with their lack of toxicity *in vivo*.

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Appendix D (pp 65-73)

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Pro-adhesive and Chemotactic Activities of Thrombospondin-1 for Breast Carcinoma Cells Are Mediated by $\alpha_3\beta_1$ Integrin and Regulated by Insulin-like Growth Factor-1 and CD98*

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Thrombospondin-1 (TSP1) is a matricellular protein that displays both pro- and anti-adhesive activities. Binding to sulfated glycoconjugates mediates most high affinity binding of soluble TSP1 to MDA-MB-435 cells, but attachment and spreading of these cells on immobilized TSP1 is primarily β_1 integrin-dependent. The integrin $\alpha_3\beta_1$ is the major mediator of breast carcinoma cell adhesion and chemotaxis to TSP1. This integrin is partially active in MDA-MB-435 cells but is mostly inactive in MDA-MB-231 and MCF-7 cells, which require β_1 integrin activation to induce spreading on TSP1. Integrin-mediated cell spreading on TSP1 is accompanied by extension of filopodia containing β_1 integrins. TSP1 binding activity of the $\alpha_3\beta_1$ integrin is not stimulated by CD47-binding peptides from TSP1 or by protein kinase C activation, which activate $\alpha_v\beta_3$ integrin function in the same cells. In MDA-MB-231 but not MDA-MB-435 cells, this integrin is activated by pertussis toxin, whereas serum, insulin, insulin-like growth factor-1, and ligation of CD98 increase activity of this integrin in both cell lines. Serum stimulation is accompanied by increased surface expression of CD98, whereas insulin-like growth factor-1 does not increase CD98 expression. Thus, the pro-adhesive activity of TSP1 for breast carcinoma cells is controlled by several signals that regulate activity of the $\alpha_3\beta_1$ integrin.

Thrombospondin-1 (TSP1)[1] is an extracellular matrix glycoprotein that has diverse effects on cell behavior (reviewed in Refs. 1 and 2). The five known thrombospondin genes display distinct patterns of expression during development and in several disease states. Disruption of the *thbs1* gene in mice results in lordosis of the spine and abnormal proliferation and inflammatory responses in the lung (3). Suppression of *THBS1* expression by loss of wild type p53, by activated Ras, Myc, nickel, and in metastatic clones of several tumor cell lines suggested that loss of TSP1 expression may contribute to tumor progression (reviewed in Ref. 4). Consistent with this hypothesis, overexpression of *THBS1* in breast carcinoma cells (5), a transformed endothelial cell line (6), fibroblasts from Li Fraumini patients (7), and glioblastoma cells (8) decreases tumor growth in animal models. This suppressive activity is due at least in part to the anti-angiogenic activity of TSP1 (reviewed in Refs. 4, 9, and 10). TSP1 antagonizes growth factor-stimulated proliferation and migration of endothelial cells. Its anti-angiogenic activity is thought to be the major mechanism for suppression of tumor growth in *THBS1*-transfected MDA-MB-435 breast carcinoma cells, because thrombospondin overexpression strongly inhibited tumor growth *in vivo* but did not significantly alter *in vitro* proliferation, motility, or the ability of the tumor cells to form colonies in soft agar (5). However, higher doses of exogenous TSP1 and some TSP1 peptides can directly inhibit proliferation of these cells *in vitro* (11).

Defining the receptors that recognize TSP1 on endothelial and tumor cells may provide insights into the differential effects of this protein on each cell type. Receptors that mediate cell interactions with TSP1 include integrins, proteoglycans, CD36, CD47, the low density lipoprotein receptor-related protein, and sulfated glycolipids. Binding of TSP1 to each of these receptors may elicit different cellular responses. Thus both the relative levels of expression of each receptor and, potentially, the activation state of each receptor may determine the nature of the adhesive, motility, and proliferative responses of cells to TSP1.

We have examined the role of integrins in the pro-adhesive activity of TSP1 for human breast carcinoma cells. Although the integrin $\alpha_v\beta_3$ is important for adhesion of several cell types to TSP1 (12), we found that adhesion of breast carcinoma cells on TSP1 substrates is not mediated by this integrin. We report here that the $\alpha_3\beta_1$ integrin rather than β_3 integrins play a dominant role in adhesion of several breast carcinoma cell lines on TSP1. The activation state of the $\alpha_3\beta_1$ integrin varies among the human breast carcinoma cell lines examined and can be modulated by inside-out signaling, suggesting that the ability to receive pro-adhesive and motility signals from TSP1 is tightly regulated in these breast carcinoma cell lines.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—Calcium-replete TSP1 was purified from human platelets as described (13). Proteolytic fragments of TSP1 were prepared as described previously (14). Synthetic peptides containing TSP1 sequences were prepared as described previously (15–17). Bovine type I collagen was obtained from Collaborative Research, and vitronectin was from Sigma. Fibronectin was purified from human plasma (National Institutes of Health Blood Bank) as described (18). Murine laminin-1 purified from the Engelbreth-Holm-Swarm sarcoma was provided by Dr. Sadie Aznavoorian. Recombinant human EGF and TGF-β1 were obtained from R & D Systems. Insulin was from Biofluids, and

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[1] The abbreviations used are: TSP1, human thrombospondin-1; BSA, bovine serum albumin; IGF1, insulin-like growth factor-1; PMA, phorbol 12-myristate 13-acetate; PT, pertussis toxin; RGD, Arg-Gly-Asp; EGF, epidermal growth factor; TGF-β, transforming growth factor-β; FGF, fibroblast growth factor; FCS, fetal calf serum; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

recombinant human insulin-like growth factor-1 (IGF1) was from Bachem.

Monoclonal Antibodies—Hybridomas producing the β_1 integrin-activating antibody TS2/16 (19) and the CD98 antibody 4F2 were obtained from the American Type Culture Collection. Antibodies secreted in PFHM-II medium (Life Technologies, Inc.) were purified by protein G affinity chromatography (Pierce). Integrin function blocking antibodies used include LM609 ($\alpha_v\beta_3$, provided by Dr. David Cheresh), 05-246 ($\alpha_1\beta_1$, Upstate Biotechnology), 6D7 ($\alpha_2\beta_1$, Dr. Harvey Gralnick, NIH), P1B5 ($\alpha_3\beta_1$, Life Technologies, Inc.), 407279 ($\alpha_4\beta_1$, Calbiochem), P1D6 ($\alpha_5\beta_1$, Life Technologies, Inc.), and mAb13 (β_1, Dr. Kenneth Yamada, NIH). Non blocking antibodies recognizing $\alpha_3\beta_1$ (M-KID2), $\alpha_4\beta_1$ (HP2/1), and $\alpha_5\beta_1$ (SAM1) were obtained from AMAC, Inc. (Westbrook, ME), and α_v (LM142) was provided by Dr. David Cheresh.

Cell Lines and Reagents—MDA-MB-435, MDA-MB-231, and MCF-7 breast carcinoma cells (American Type Culture Collection) were grown in RPMI 1640 medium containing 10% FCS. Okadaic acid, PMA, pertussis toxin (PT), herbimycin A, heparin, and sodium vanadate were purchased from Sigma. Pertussis toxin B oligomer, staurosporine, wortmannin, KT5823, guanosine-3′,5′-cyclic monophosphothioate, 8-(4-chlorophenylthio)-, *Rp* isomer, and bisindolylmaleimide were from Calbiochem. KT5720 was from Kamiya Biomedical (Thousand Oaks, CA).

Adhesion Assays—Cells were detached by replacing the growth medium with PBS containing 2.5 mM EDTA and incubating 5–10 min at 37 °C. The cells were collected by centrifugation, suspended in RPMI containing 0.1% BSA, and assayed for adhesion to bacteriological polystyrene substrates coated with proteins as described previously (14). Adhesion assays were terminated after 50 min by washing to remove nonadherent cells and fixation with 1% glutaraldehyde in PBS.

Chemotaxis—Chemotaxis was measured in 48-well chambers using Nucleopore 8 μm, polyvinylpyrrolidone-free filters (Neuroprobe Inc, Gaithersburg, MD). To provide an integrin-independent substrate for motility, the filters were coated with 10 μg/ml polylysine for 16 h at 4 °C prior to use. Motility was measured after 6.5 h and scored microscopically by counting nuclei of migrated cells on the lower surface of the membrane.

Fluorescence Microscopy—To examine integrin localization and cytoskeletal rearrangement, 8-well glass chamber slides (Nalge Nunc International, Naperville, IL) were coated with type I collagen, TSP1, or fibronectin overnight at 4 °C. The chambers were then blocked with 1% BSA in PBS, and cells were added in RPMI containing 0.1% BSA. In some cases, antibodies were included in the medium. Cells were allowed to attach and spread for 90 min. The unbound cells were then removed along with the medium, and the chambers were rinsed with PBS and fixed with 3.7% formaldehyde. Cells were stained with BODIPY TR-X phallacidin (Molecular Probes, Inc. Eugene, OR) to visualize F-actin or using primary antibodies followed by BODIPY FL anti-mouse IgG to localize integrins or CD98. All staining procedures were carried out according to the manufacturer's directions. Stained cells were observed and photographed under a Zeiss fluorescent microscope using appropriate filters.

Unstimulated MDA-MB-435 cells were evaluated for expression of integrins or their subunits 1 day after plating in RPMI medium containing 10% FCS (Biofluids) by indirect immunofluorescence and flow cytometry. Cells were washed with PBS, 0.2% BSA and incubated at 37 °C for 6 min with Puck's saline A with 0.2% EDTA and 10% FCS. All subsequent procedures were performed on ice, and all washes were with PBS containing 0.2% BSA. Cells were dislodged with a scraper, and the resultant cell suspension was washed. Cell pellets were exposed to mouse IgG or primary antibodies to integrins or integrin subunits in PBS, 0.2% BSA, washed, and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Tago, Inc., Burlingame, CA). Following a wash, the cells were fixed in 1% paraformaldehyde and analyzed in a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Initial gating was done using forward and side scatter to identify a population of intact cells without debris.

Ligand Binding—TSP1 was iodinated using Iodogen (Pierce) as described previously (20). For some experiments, cells were grown in sulfate-deficient medium containing chlorate to inhibit proteoglycan and glycolipid sulfation as described previously (21).

RESULTS

Integrin Expression on Breast Carcinoma Cells—Flow cytometric analysis (Table I) and immunoprecipitation using subunit-specific integrin antibodies (data not shown) demonstrated that MDA-MB-435 cells express several β_1 integrins and $\alpha_v\beta_3$. Integrin expression on MDA-MB-231 and MCF-7 cells has been reported previously (22–24). MDA-MB-231 cells express α_2, α_3, α_4, α_5, α_6, α_v, and β_1 subunits. The MDA-MB-231 and MCF-7 cell lines express only low levels of β_3 subunits (24).

TABLE I
Integrin expression in MDA-MB-435 breast carcinoma cells

Integrin	Antibody	Mean fluorescence intensity
$\alpha_2\beta_1$	6D7	200
$\alpha_3\beta_1$	M-KID2	127
$\alpha_4\beta_1$	HP2/1	98
$\alpha_5\beta_1$	SAM1	122
α_v	LM142	256
$\alpha_v\beta_3$	LM609	157
β_1	mAb13	158
	Mouse IgG	31

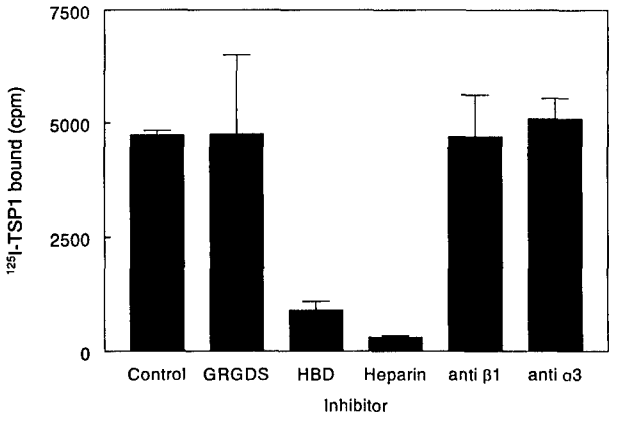

FIG. 1. **Specificity of ^{125}I-thrombospondin binding to MDA-MB-435 cells.** Cells were harvested using 2.5 mM EDTA in PBS, resuspended in RPMI 1640 medium containing 0.1% BSA, and incubated with ^{125}I-thrombospondin for 1 h at 25 °C with the indicated inhibitors. Cells were centrifuged through oil to remove unbound labeled protein. The mean ± S.D. for triplicate determinations is presented for binding determined in the absence (control) or presence of presence of 204 μM GRGDS peptide, 4 μM 18-kDa recombinant TSP1 heparin-binding domain (*HBD*), 100 μg/ml heparin, 10 μg/ml mAb13 (anti-β_1) or P1B5 (anti-$\alpha_3\beta_1$).

Binding of Soluble TSP1—Previous studies using MDA-MB-231 breast carcinoma cells (25) concluded that sulfated glycoconjugates including heparan sulfate and chondroitin sulfate proteoglycans play a dominant role in both binding of soluble TSP1 and adhesion on immobilized TSP1. We observed a similar dependence for ^{125}I-TSP1 binding to MDA-MB-435 cells (Fig. 1). Binding was strongly inhibited by heparin or a recombinant 18-kDa amino-terminal heparin-binding fragment of TSP1, but the peptide GRGDS and β_1 or α_3 integrin function blocking antibodies had no effect. Conversely, binding of ^{125}I-TSP1 to MDA-MB-435 cells was not enhanced by incubation with the β_1 integrin-activating antibody TS2/16, either alone or in the presence of 10 μg/ml heparin to inhibit TSP1 binding to sulfated ligands (data not shown). Therefore, high affinity binding to soluble TSP1 to these cells is mediated by sulfated glycoconjugates and is independent of integrin binding.

β_1 Integrin-mediated Adhesion and Chemotaxis to TSP1—Although heparin and recombinant heparin binding domain from TSP1 partially inhibited attachment of MDA-MB-435 cells on immobilized TSP1, the fraction of spread cells was unaffected (Fig. 2*A*). In the presence of a β_1 integrin function blocking antibody at 2 μg/ml, however, only spreading was inhibited, and a combination of heparin and the β_1 blocking antibody abolished spreading and markedly inhibited attachment. At 50 μg/ml, the β_1 antibody completely inhibited adhesion to TSP1 (Fig. 2*A*). Thus, interaction with a β_1 integrin is essential for spreading, but sulfated ligands may also contrib-

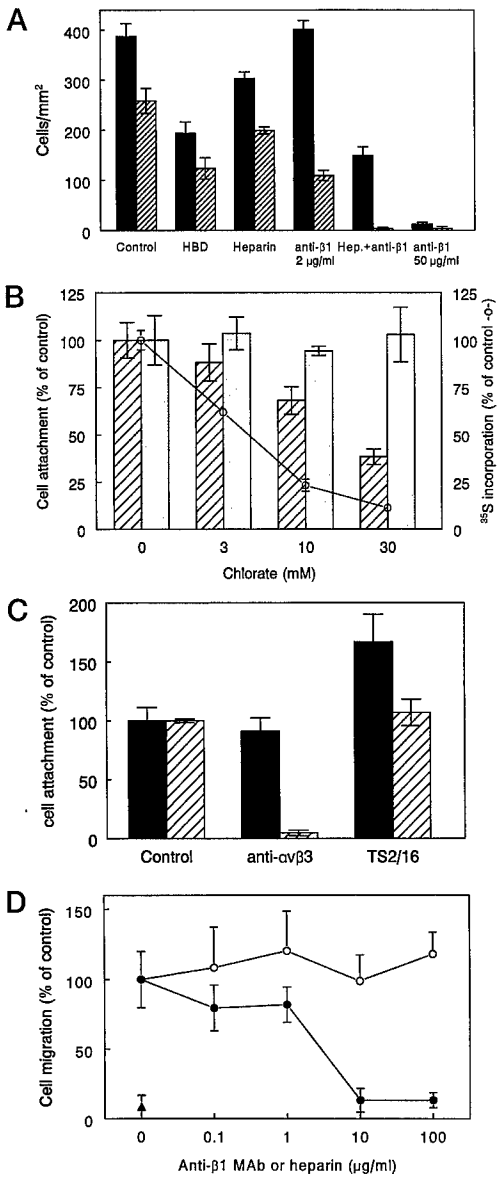

FIG. 2. **Role of integrins and sulfated glycoconjugates in breast carcinoma cell adhesion and chemotaxis to TSP1.** *A,* MDA-MB-435 cell attachment (*solid bars*) and spreading (*striped bars*) were measured after 50 min on polystyrene coated with thrombospondin (50 μg/ml) and blocked with 1% BSA to reduce nonspecific adhesion. Heparin-dependent adhesion was assessed by inhibition using 4 μM 18-kDa recombinant TSP1 heparin-binding domain (*HBD*) or 50 μg/ml heparin. β_1 integrin-dependent adhesion was inhibited using 2 or 50 μg/ml mAb13 (anti-β_1). Results are presented as mean ± S.D., $n = 3$. *B,* effect of inhibiting sulfation on attachment of MDA-MB-435 cells. MDA-MB-435 cells were grown in Ham's F-12 medium (low sulfate) containing 10% dialyzed fetal calf serum for 48 h. The medium was replaced with the same medium containing 1% dialyzed serum with or without sodium chlorate at the indicated concentrations. The cells were cultured for 24 h, harvested, and resuspended in F-12 medium containing 1 mg/ml BSA with or without chlorate at the indicated concentrations. Cell adhesion was quantified to polystyrene coated with 50 μg/ml thrombospondin (*striped bars*) or 10 μg/ml fibronectin (*gray bars*). ^{35}S incorporation in MDA-435 cell macromolecules (○) was assessed in duplicate cultures supplemented with 25 μCi/ml [^{35}S]sulfate. The cells were fixed and washed in acetic acid/methanol, and incorporation of radioactivity in macromolecules was determined by scintillation counting after solubilization in 1% sodium dodecyl sulfate. *C,* integrin $\alpha_v\beta_3$ mediates breast carcinoma cell adhesion to vitronectin but not to TSP1. Adhesion of MDA-MB-435 cells to 30 μg/ml TSP1 (*solid bars*) or 10 μg/ml vitronectin (*striped bars*) was measured in the presence of the $\alpha_v\beta_3$ function blocking antibody LM609 or the β_1-activating antibody TS2/16. *D,* chemotaxis to TSP1 is β_1 integrin-dependent. MDA-MB-435 chemotaxis to 50 μg/ml TSP1 was determined in modified Boyden chambers. Cells were added in the upper chamber with the indicated concentrations of β_1 integrin blocking antibody mAb13 (●) or heparin (○). Spontaneous motility (▲) was determined in the absence of TSP1. Migrated cells were counted microscopically, and results from triplicate wells are presented as a percent of migration to TSP1 without inhibitors, mean ± S.D.

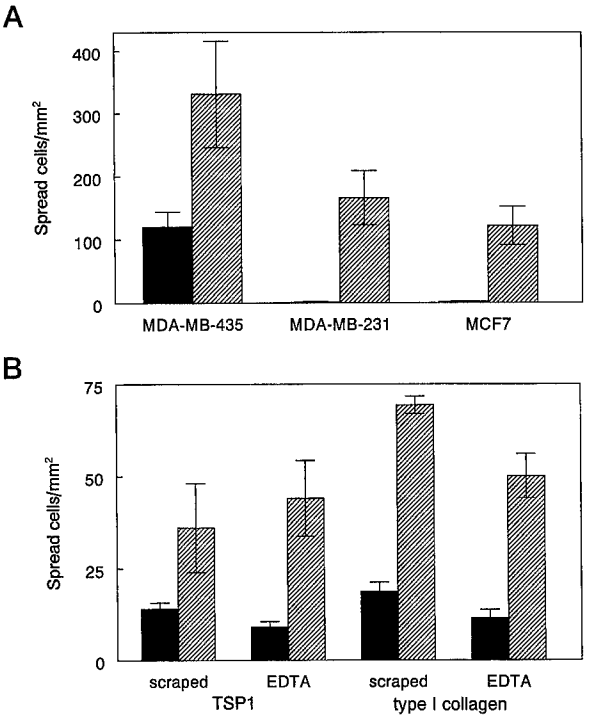

FIG. 3. **β_1 integrins recognizing TSP1 and type I collagen are partially inactive in human breast carcinoma cell lines.** *A,* spreading of three breast carcinoma cell lines on 50 μg/ml TSP1 (*solid bars*) or on TSP1 in the presence of 5 μg/ml TS2/16 (*striped bars*). *B,* comparison of β_1 integrin activity in MDA-MB-435 cells harvested by scraping in RPMI medium or by a 5-min treatment with 2.5 mM EDTA in PBS. Cells were resuspended in RPMI medium with 0.1% BSA (*solid bars*) or with 20 μg/ml TS2/16 (*striped bars*), and cell spreading was assessed after 50 min on substrates coated with 20 μg/ml TSP1 or 5 μg/ml type I collagen.

ute to adhesion of these cells on TSP1. This was confirmed by inhibition of sulfation following growth in chlorate. Adhesion was inhibited by 60% for MDA-MB-435 cells with a 90% reduction in $^{35}SO_4$ incorporation (Fig. 2*B*). RGD peptides did not inhibit adhesion of MDA-MB-435 cells on TSP1 (results not shown).

Although MDA-MB-435 cells express some $\alpha_v\beta_3$ integrin (Table I), a function blocking antibody or an $\alpha_v\beta_3$-specific RGD mimetic blocked adhesion of the cells on vitronectin but had no effect on adhesion on TSP1 (Fig. 2*C* and results not shown). Conversely, in the presence of the β_1-activating antibody TS2/16, adhesion of MDA-MB-435 cells was enhanced on TSP1 but not on vitronectin (Fig. 2*C*). Therefore, the $\alpha_v\beta_3$ integrin is functional in MDA-MB-435 cells, but it is apparently unable to recognize the RGD motif in TSP1.

The β_1 blocking antibody mAb13 inhibited chemotaxis to TSP1, but heparin did not (Fig. 2*D*). For these experiments, the filters were coated with polylysine to provide an integrin-independent substrate for adhesion of the cells. Therefore, chemotaxis of MDA-MB-435 cells to TSP1 is also primarily dependent on the β_1 integrin receptor.

Several human breast cancer cell lines showed similar involvement of β_1 integrins in their adhesion to TSP1 (Fig. 3). MDA-MB-231 cells attached poorly and did not spread on substrates coated with low concentrations of TSP1. In the presence of the β_1-activating antibody, however, the cells attached av-

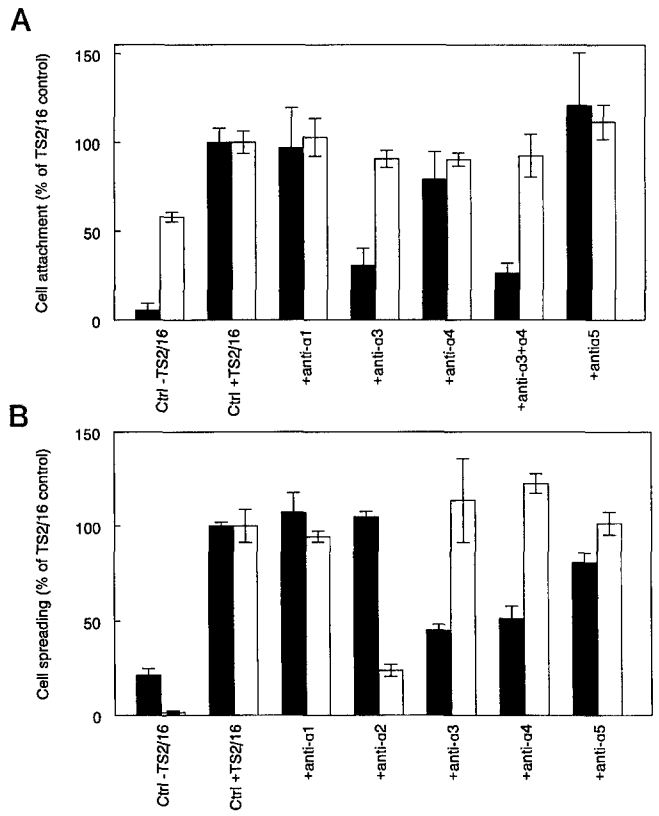

FIG. 4. **Integrin α subunit specificity of TSP1 adhesion.** *A*, MDA-MB-231 cell attachment was quantified using substrates coated with 40 μg/ml TSP1 (*solid bars*) or 5 μg/ml type I collagen (*gray bars*) in the presence of 5 μg/ml TS2/16 to activate β_1 integrins and 5 μg/ml of the indicated function blocking antibodies. *B*, inhibition of MDA-MB-435 cell spreading on TSP1 (*solid bars*) or type I collagen (*gray bars*) in the presence of TS2/16 and the indicated α subunit blocking antibodies.

idly on TSP1 and exhibited spreading (Fig. 3*A*). A third breast carcinoma cell line, MCF-7, behaved similarly to the MDA-MB-231 cells and showed spreading on TSP1 only in the presence of the β_1-activating antibody (Fig. 3*A*). The apparent low avidity state of the integrin that recognizes TSP1 on MDA-MB-435 cells was not an artifact from using EDTA to dissociate the cells, because cells suspended by scraping from the dish in the presence of divalent cations showed the same degree of enhancement by TS2/16 for adhesion to TSP1 or type I collagen as cells harvested using EDTA (Fig. 3*B*).

$\alpha_3\beta_1$ Is the Major TSP1-binding Integrin on Breast Carcinoma Cells—Of the α subunit antibodies tested for inhibiting adhesion to TSP1, only an α_3 subunit blocking antibody, P1B5, significantly inhibited adhesion of MDA-MB-231 cells to TSP1 (Fig. 4*A*, $p = 0.0007$, 2-tailed t test). An α_4 integrin blocking antibody slightly inhibited adhesion, but mixing this antibody with the α_3 blocking antibody produced no further inhibition than the latter antibody alone (Fig. 4*A*). MDA-MB-435 cell spreading on TSP1 was also inhibited by the α_3 blocking antibody, and somewhat by the α_4 antibody (Fig. 4*B*). Function blocking antibodies for $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_5\beta_1$ integrins had no effect on TSP1 adhesion, although the $\alpha_2\beta_1$ and $\alpha_5\beta_1$ antibodies inhibited adhesion of the same cells to known ligands for these integrins (Fig. 4 and results not shown).

Integrin Localization and Effects on Actin Cytoskeleton—Activation of β_1 integrins using TS2/16 altered the morphology of cells attaching on TSP1 (Fig. 5). MDA-MB-435 cells extended a few processes but exhibited no F-actin organization when attached on TSP1 alone (Fig. 5*a*), but addition of antibody TS2/16 stimulated spreading with redistribution of F-actin to the cell periphery (Fig. 5*b*). F-actin was also present in short spikes protruding from the spread cells but did not organize into stress fibers. Staining with the β_1 integrin antibody revealed numerous filopodia extending from these points (Fig. 5*c*). In some cells, these filopodia were terminated with punctate β_1 integrin staining, possibly at sites of contact with the TSP1 substrate. Formation of filopodia was specific to the TSP1 substrate, as TS2/16-induced spreading of these cells on type I collagen (Fig. 5*d*) or fibronectin (results not shown) only rarely evoked filopodia. These cytoskeletal rearrangements were specific for β_1-dependent adhesion to intact TSP1 and were not observed in cells attaching on heparin-binding peptides or recombinant fragments of TSP1 (results not shown). Similar induction of filopodia or microspikes by TSP1 have been observed in other cell types (26).

Conformation Requirements for $\alpha_3\beta_1$-Mediated Adhesive Activity of TSP1—Differences in the conformation or folding of TSP1 could account for discrepancies in its reported adhesive activity. The conformation of TSP1 and formation of specific intra-chain disulfide bonds are sensitive to the levels of divalent cations present during its purification. Disulfide bonding also influences interactions of TSP1 with several proteases and regulates the accessibility of the RGD sequence to the $\alpha_v\beta_3$ integrin (27, 28). We therefore examined the influence of conformation on $\alpha_3\beta_1$-dependent adhesion by absorbing TSP1 with or without divalent cations, at low pH (29), or by reducing disulfide bonds using dithiothreitol (Fig. 6). Coating TSP1 at pH 4 in acetate buffer enhanced MDA-MB-435 cell adhesion relative to TSP1 adsorbed in PBS with Ca^{2+} and Mg^{2+}, but use of PBS with 2.5 mM EDTA did not significantly affect β_1-mediated adhesion. Although heparin only partially inhibited MDA-MB-435 cell adhesion to TSP1 (20–50%) when the TSP1 was adsorbed in Dulbecco's PBS (*e.g.* Fig. 2*A*), adhesion to TSP1 adsorbed in pH 4 acetate buffer was inhibited 98% by 10 μg/ml heparin. Conversely, TS2/16 did not reproducibly increase adhesion of MDA-MB-435 cells to TSP1 adsorbed in acetate buffer (data not shown). Therefore, the enhanced adhesion to thrombospondin coated at pH 4 was due primarily to enhancement of heparin-dependent adhesion, whereas β_1-integrins contributed less to adhesion on TSP1 coated at the lower pH. Adhesion of MDA-MB-435 cells (Fig. 6) and MDA-MB-231 cells (results not shown) was strongly inhibited following reduction of TSP1 with dithiothreitol. This contrasts with $\alpha_v\beta_3$-dependent adhesion to TSP1, which was reported to be enhanced following disulfide reduction using the same conditions as used in Fig. 6 (28). Thus, $\alpha_3\beta_1$-dependent adhesion of breast carcinoma cells does not require Ca^{2+}-replete TSP, but some intact disulfide bonds are essential.

Regulation of β_1 Integrin Activation in Breast Carcinoma Cells—Adhesion of T lymphocytes to TSP1, mediated by $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, is stimulated by phorbol esters (30). PMA activation of protein kinase C in MDA-MB-435 cells increased $\alpha_v\beta_3$-mediated adhesion to vitronectin but had no effect on β_1 integrin-mediated adhesion to TSP1 (Fig. 7). Integrin-associated protein (CD47) also regulates integrin function in several cell types (31, 32). The carboxyl-terminal domain of TSP1 contains two peptide motifs that activate integrin function through binding to CD47 (31). The CD47-binding TSP1 peptide 7N3 activated adhesion of MDA-MB-435 cells on vitronectin (Fig. 7) and a recombinant TSP1 fragment containing the RGD sequence (results not shown) but had no effect on adhesion to native TSP1 (Fig. 7). Thus MDA-MB-435 cells express functional $\alpha_v\beta_3$ that can be activated by PMA or the TSP1 7N3 peptide. This $\alpha_v\beta_3$ integrin can recognize the TSP1 RGD sequence in the context of a bacterial fusion protein, but it does not play a significant role in adhesion of resting or stimulated breast carcinoma cells to native platelet TSP1.

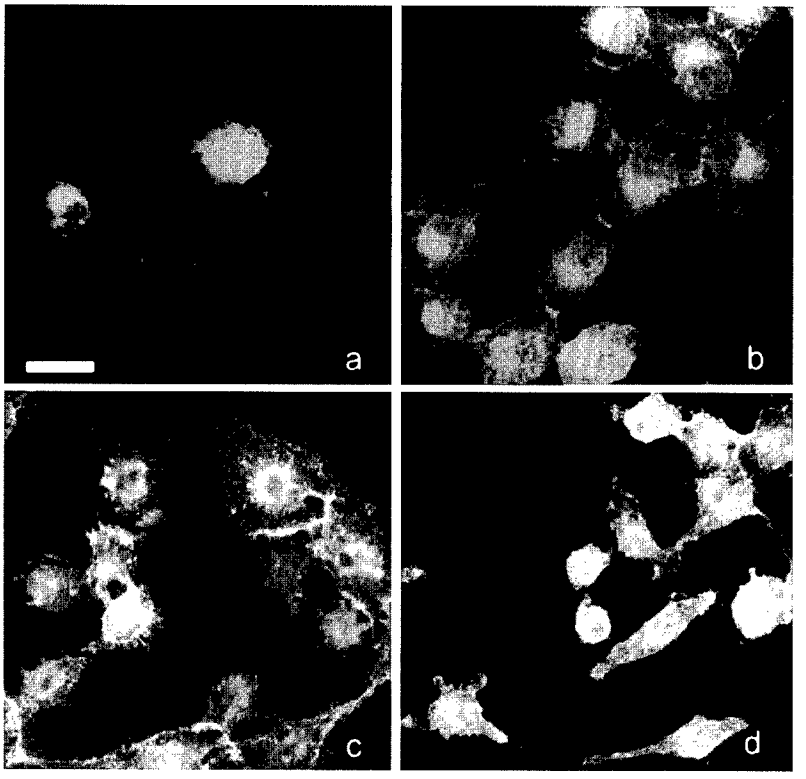

FIG. 5. **Actin organization and filopodia formation on TSP1 is stimulated by β_1 integrin activation.** Actin was visualized using BODIPY-phallacidin in MDA-MB-435 cells attached on TSP1 (*a*) or TSP1 in the presence of 5 μg/ml TS2/16 (*b*). β_1 integrin localization of the TS2/16-treated cells was visualized using BODIPY FL-anti-mouse IgG on TSP1 (*c*) or type I collagen substrates (*d*). *Bar* in *a* = 20 μm.

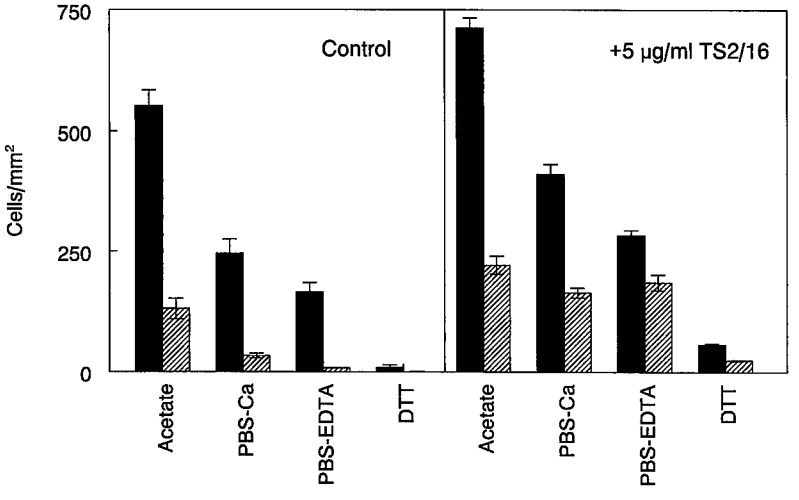

FIG. 6. **Tertiary structure dependence for MDA-MB-435 cell adhesion on TSP1.** MDA-MB-435 cell adhesion to 20 μg/ml TSP1 coated on polystyrene in 10 mM sodium acetate, 150 mM NaCl, pH 4 (29), Dulbecco's PBS with calcium and magnesium (PBS-Ca^{2+}), PBS with 2.5 mM EDTA, or PBS with 2.5 mM EDTA and 2 mM dithiothreitol (DTT). Attachment (*solid bars*) and spreading (*striped bars*) were assessed in the absence or presence of 5 μg/ml TS2/16.

Several pharmacological agents stimulated β_1-dependent adhesion to TSP1 (Table II). The broad spectrum Ser/Thr protein kinase inhibitor staurosporine increased spreading of all three cell lines. However, this activation in MDA-MB-435 cells was only partially replicated by specific inhibitors of protein kinase C (bisindolylmaleimide), protein kinase A (KT5720), or protein kinase G (KT5823 and guanosine-3′,5′-cyclic monophosphorothioate, 8-(4-chloro-phenylthio)-, *R*p isomer). Inhibition of phosphatidylinositol 3-kinase using wortmannin had no significant effect on MDA-MB-435 cell spreading and weakly enhanced MDA-MB-231 cell spreading on TSP1. Two calcium ionophores, ionomycin and A23187, strongly enhanced spreading of MDA-MB-435 cells but had no effect on MDA-MB-231 cell spreading on TSP1.

Modulation of TSP1 Adhesion by G-protein Signaling—Although TSP1 peptides promote PT-sensitive integrin activation through binding to CD47 (31, 33), we showed above that this pathway does not function in MDA-MB-435 cells to activate $\alpha_3\beta_1$. However, PT did influence MDA-MB-231 and MDA-MB-435 cell adhesion and spreading on TSP1 or collagen (Fig. 8). PT increased adhesion of MDA-MB-231 cells to TSP1 (Fig. 8*A*) but inhibited both basal and TS2/16-stimulated adhesion of MDA-MB-435 cells on the same substrate (Fig. 8*B*). The effects of PT in both cell lines were specific, since PT B-oligomer at the same concentration had no effect (Fig. 8). The enhancement of MDA-MB-231 cell adhesion by PT is mediated by the β_1 integrin, because the β_1 blocking antibody mAb13 inhibited the PT-induced adhesion of MDA-MB-231 cells but heparin did not (results not shown). However, not all β_1 integrins in these breast carcinoma cells were activated by PT. Adhesion of MDA-MB-231 cells to collagen mediated by $\alpha_2\beta_1$ (verified by the blocking antibody 6D7, results not shown) was not altered by PT, although the same adhesive pathway could be further activated by TS2/16 (Fig. 8*A*). In MDA-MB-435 cells, PT partially inhibited $\alpha_2\beta_1$-mediated spreading on collagen stimulated by TS2/16 (Fig. 8*B*).

Physiological Activators of TSP1 Adhesion and Chemotaxis—We noted that freshly passaged breast carcinoma cells exhibited stronger β_1 integrin-mediated adhesion on TSP1. This suggested that proliferation regulates $\alpha_3\beta_1$-mediated TSP1 ad-

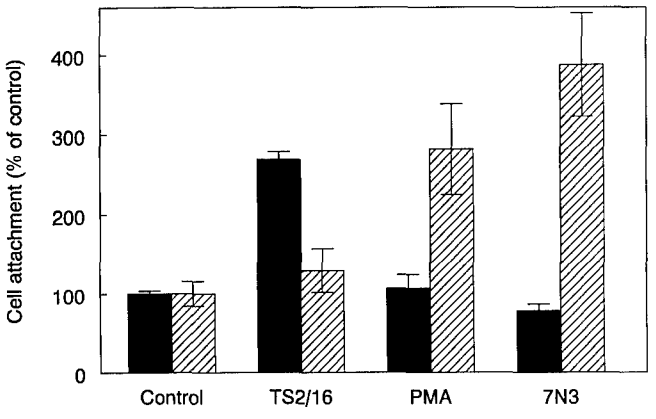

FIG. 7. **Differential regulation of β_1 and β_3 integrin activity in MDA-MB-435 cells.** Attachment of MDA-MB-435 cells on 5 μg/ml vitronectin (*striped bars*) or 40 μg/ml TSP1 (*solid bars*) was measured using cells treated with 20 μg/ml TS2/16, 10 ng/ml PMA, or 3 μM of the CD47-binding TSP1 peptide 7N3 (FIRVVMYEGKK). Results are presented as a percentage of cell attachment without additions, mean ± S.D., $n = 3$.

TABLE II
Modulation of breast carcinoma spreading on TSP1

MDA-MB-435 or MDA-MB-231 cell spreading on TSP1 was measured in untreated cells in the presence or absence of the β_1-activating antibody TS2/16 to measure basal and total β_1-dependent adhesion and in cells pretreated with and maintained in the following inhibitors: 10 nM staurosporine (Ser/Thr kinase inhibitor), 100 nM KT5720 (protein kinase A), 200 nM bisindolylmaleimide (protein kinase C), 1 μM KT5823 or 2 μM guanosine-3′,5′-cyclic monophosphorothioate, 8-(4-chlorophenylthio)-, *R*p isomer (protein kinase G), 2 nM wortmannin (phosphatidylinositol 3-kinase), 1 μg/ml ionomycin or A23187 (calcium ionophores), 1 μM herbimycin (tyrosine kinase), or 20 μM vanadate. The net increase in cell spreading in the presence of the indicated drugs is expressed as a percent of that induced by the β_1-activating antibody TS2/16, mean ± S.D., $n = 3$.

Inhibitor	Cell line		
	MDA-MB-435	MDA-MB-231	MCF-7
	spreading (% TS2/16)		
Staurosporine	112 ± 16	13 ± 3	39 ± 8
KT5720	35 ± 25	1 ± 2	
Bisindolylmaleimide	15 ± 10	0 ± 1	
KT5823	17.2 ± 7.9	0 ± 1	
RP8-pCPT-cGMPS	−2 ± 6	3 ± 1	
Wortmannin-2	−18 ± 18	16 ± 4	
Ionomycin	52 ± 3	0 ± 0	
A23187	83 ± 29	0 ± 0	
Herbimycin	−16 ± 1	0 ± 1	
Vanadate	−9 ± 2	8 ± 2	

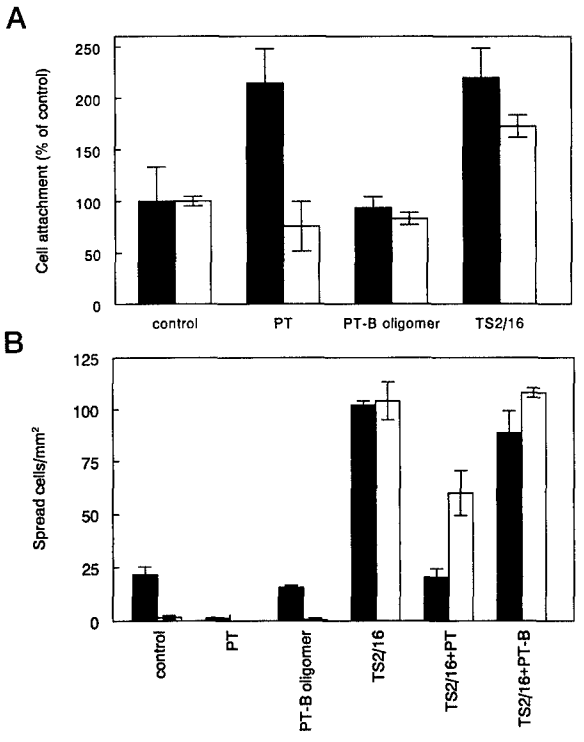

FIG. 8. **Pertussis toxin differentially regulates MDA-MB-435 and MDA-MB-231 cell adhesion on TSP1.** *A*, MDA-MB-231 cell attachment on 40 μg/ml TSP1 (*solid bars*) or 5 μg/ml type I collagen (*gray bars*) was measured alone or in the presence of 5 μg/ml TS2/16, 1 μg/ml PT, or 1 μg/ml PT B-oligomer. Results are mean ± S.D. for triplicate determinations. *B*, MDA-MB-435 cell spreading was determined on TSP1 (*solid bars*) or type I collagen substrates (*gray bars*) in the presence of PT or PT B-oligomer added alone or combined with 5 μg/ml TS2/16.

hesion. Serum induced a dose-dependent increase in β_1 integrin-mediated attachment (Fig. 9*A*) and spreading of MDA-MB-435 cells to TSP1 or type I collagen. A similar serum response was observed in MDA-MB-231 cells for adhesion on TSP1, although adhesion of the latter cell line to type I collagen was maintained in the absence of serum (data not shown).

Several growth factors were examined to define the basis of the serum response for TSP1 adhesion (Fig. 9*B*). Addition of EGF to serum-depleted medium increases adhesion of breast carcinoma cells to some substrates (34) but in several experiments showed only a slight stimulatory activity for spreading of MDA-MB-435 cells on TSP1 (Fig. 9*B*). FGF2 and TGF-β1 were also ineffective, but addition of insulin stimulated MDA-MB-435 cell adhesion to a greater extent than 10% serum (Fig. 9*B*). Insulin was also the only growth factor tested that stimulated adhesion of MDA-MB-231 cells to TSP1 (results not shown).

Acute addition of insulin, but not EGF, during the adhesion assay produced a similar enhancement in adhesion of both cell lines to TSP1 as the 24-h pretreatment of the cells in culture (Fig. 9*C* and results not shown). The dose dependence for the insulin response was consistent with that for signaling through the IGF1 receptor (Fig. 9*C*), which is expressed in these breast carcinoma cells (35). Both insulin and IGF1 strongly stimulated MDA-MB-435 cell spreading on TSP1, moderately stimulated adhesion on type I collagen, but did not stimulate adhesion on laminin-1 (Fig. 9*C*). EGF (2 nM) was inactive in this assay (results not shown). IGF1 (EC_{50} = 1 nM) was 100-fold more potent than insulin, as expected for a response mediated by the IGF1 receptor (35). A similar difference in the potencies of IGF1 and insulin was also observed in stimulation of TSP1 attachment of MDA-MB-231 cells (results not shown). Thus, occupancy of the IGF1 receptor specifically stimulates activity of the TSP1-binding integrin in both cell lines.

IGF1 also enhanced the chemotactic response of breast carcinoma cells to TSP1. Addition of IGF1 to MDA-MB-435 cells in the upper well of a modified Boyden chamber did not alter motility of the cells, but it stimulated (2- to 5-fold) the chemotactic response to TSP1 added to the lower chamber (Fig. 9*D*). This IGF1-stimulated motility to TSP1 was mediated by the $\alpha_3\beta_1$ integrin, because mAb13 (anti-β_1) and P1B5 antibodies (anti-α_3) strongly inhibited direct TSP1 chemotaxis and that stimulated by IGF1. IGF1-stimulated chemotaxis to TSP1 was also sensitive to PT inhibition (Fig. 9*D*).

Modulation of TSP1 Adhesion by CD98—Expression of the transmembrane protein CD98 is induced by serum, and this protein was recently shown to activate function of some β_1 integrins (36). Clustering of CD98 using the antibody 4F2 stimulates small cell lung carcinoma adhesion on fibronectin and laminin (36) and similarly activated $\alpha_3\beta_1$-mediated spreading of breast carcinoma cells on TSP1 and $\alpha_2\beta_1$-mediated

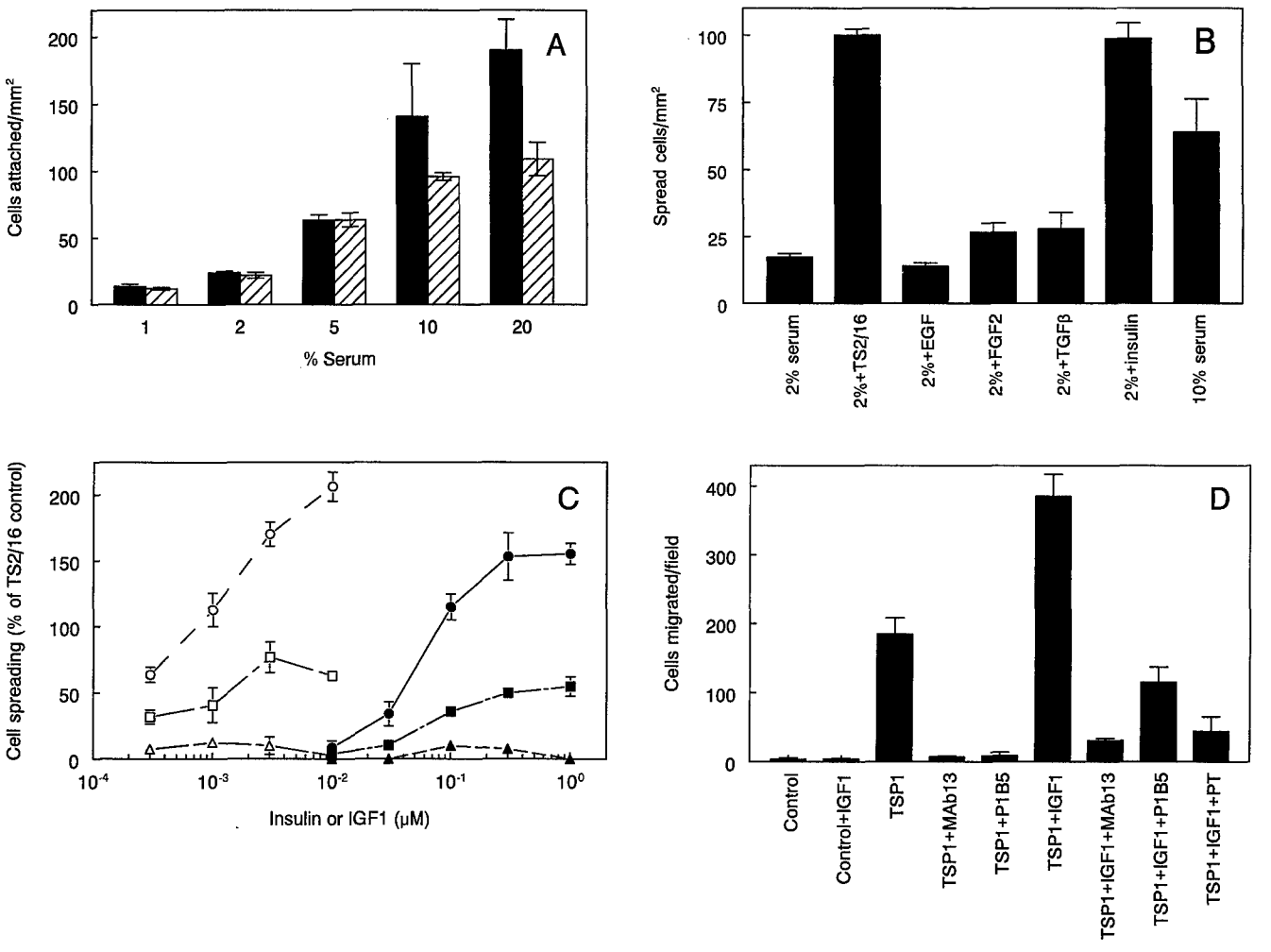

FIG. 9. **Regulation of β_1 integrin-mediated TSP1 interactions by serum and growth factors.** *A,* serum induces attachment of MDA-MB-435 cells to TSP1 (*solid bars*) and type I collagen (*striped bars*). Cells were grown for 24 h in RPMI medium containing the indicated concentration of FCS. *B,* insulin specifically induces adhesion of breast carcinoma cells to TSP1. MDA-MB-435 cell spreading on surfaces coated with 40 μg/ml TSP1 was determined using cells grown for 24 h in RPMI medium containing 2% serum and supplemented with the indicated growth factors (10 ng/ml EGF, 100 ng/ml FGF2, 5 ng/ml TGF-β, or 10 μg/ml insulin) or RPMI medium containing 10% serum. Spreading of cells grown in 2% serum was also tested in the presence of 5 μg/ml antibody TS2/16 (2% + TS2/16) to assess maximal β_1 integrin-mediated spreading activity. *C,* dose dependence for induction of TSP1 adhesion by insulin and IGF1. Cell spreading after 50 min (expressed as a percentage of maximal spreading elicited on each substrate in the presence of 5 μg/ml TS2/16 antibody) was determined in RPMI medium containing 0.1% BSA and supplemented with the indicated concentrations of insulin (*closed symbols*) or IGF1 (*open symbols*) using substrates coated with 40 μg/ml TSP1 (○ and ●), 20 μg/ml laminin (▲ and △), or 5 μg/ml type I collagen (■ and □). *D,* IGF1 synergizes with TSP1 to promote chemotaxis of MDA-MB-435 cells. Chemotaxis to 50 μg/ml TSP1 was determined in the presence of the indicated inhibitors or stimulators at the following concentrations: 10 nM IGF1 5 μg/ml mAb13 (anti-β_1), 5 μg/ml P1B5 (anti-α_3), and 1 μg/ml PT. Results are mean ± S.D., n = 3–6.

adhesion on type I collagen (Fig. 10*A* and results not shown). Induction of $\alpha_3\beta_1$-mediated TSP1 adhesion in serum-containing growth medium may be mediated by induction of CD98 expression, because a 24-h exposure to 10% serum increased surface expression of CD98 in MDA-MB-435 cells (Fig. 10*B*). IGF1 treatment for the same time, however, decreased CD98 expression (Fig. 10*B*), indicating that increased CD98 expression does not mediate the response to IGF1.

DISCUSSION

The $\alpha_3\beta_1$ integrin, with some cooperation of sulfated glycoconjugates and $\alpha_4\beta_1$ integrin, mediates adhesion of MDA-MB-435 and MDA-MB-231 breast carcinoma cells to TSP1. This β_1 integrin is maintained in an inactive or partially active state in these cell lines but can be activated by exogenous stimuli including serum, insulin, IGF1, and ligation of CD98. In MDA-MB-231 cells, the inactive state of the $\alpha_3\beta_1$ integrin is maintained by a G-protein-mediated signal, but this suppression can also be overcome by IGF1 receptor signaling. Stimuli that increase β_1-dependent adhesion to TSP1 do not stimulate β_3-dependent adhesion to TSP1, even though the cells express the known TSP1 receptor $\alpha_v\beta_3$, and this integrin is functional and inducible for vitronectin adhesion. We do not know why the $\alpha_v\beta_3$ integrin on MDA-MB-435 cells cannot recognize the RGD sequence in the type III repeat of platelet TSP1. Other cell types, however, can utilize the same TSP1 preparations used for these experiments to support $\alpha_v\beta_3$-dependent adhesion.[2]

Several β_1 integrins have been implicated as TSP1 receptors in other cell types, including $\alpha_2\beta_1$ on activated platelets (37), $\alpha_3\beta_1$ on neurons (38), and $\alpha_4\beta_1$ and $\alpha_5\beta_1$ on activated T lymphocytes (30). $\alpha_3\beta_1$ is the dominant integrin for mediating adhesive activity of breast carcinoma cells for TSP1, whereas $\alpha_2\beta_1$ mediates adhesion of these cells to type I collagen but not to TSP1. The integrin $\alpha_4\beta_1$ may play a role in adhesion of some breast carcinoma cell lines to TSP1, as we previously reported for T lymphocytes (30). The mechanism for the apparent dif-

[2] J. M. Sipes, H. C. Krutzsch, J. Lawler, and D. D. Roberts, submitted for publication.

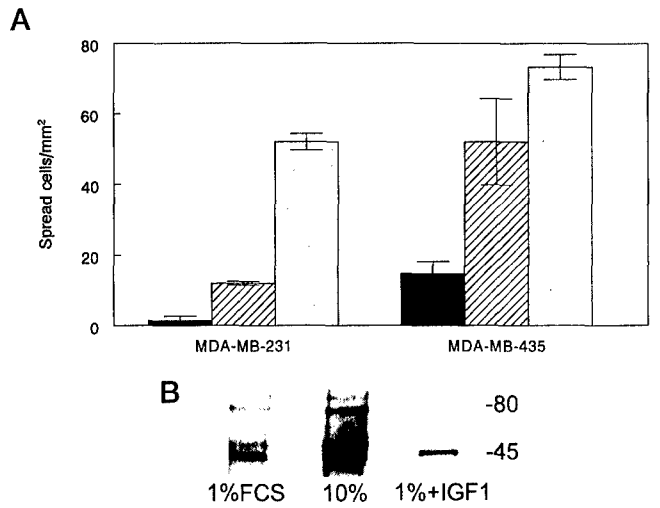

FIG. 10. **CD98 ligation stimulates breast carcinoma cell adhesion to TSP1.** *A*, basal (*solid bars*) or stimulated MDA-MB-231 or MDA-MB-435 cell spreading on 25 μg/ml TSP1 was determined in the presence of 5 μg/ml TS2/16 (*striped bars*) or 20 μg/ml 4F2 (*gray bars*). *B*, serum induces but IGF1 inhibits CD98 expression. MDA-MB-435 cells grown 24 h in RPMI medium containing 1% FCS, 10% FCS, or 1% FCS and 10 nM IGF1 as described in *A* were biotinylated, and equal amounts of cell protein were immunoprecipitated with antibody 4F2. The immunoprecipitates were analyzed by SDS-gel electrophoresis and Western blotting using streptavidin-peroxidase and chemiluminescent detection. Markers indicate the migration of the 80- and 45-kDa subunits of CD98.

ferential recognition of TSP1 by β_1 integrins among these cell types remains to be defined. However, it is notable that even within the breast carcinoma cell lines, pharmacological and physiological stimuli can differentially modulate activity of the $\alpha_3\beta_1$ integrin for promoting adhesion or chemotaxis to TSP1. This finding implies a complex signaling process that regulates the recognition of pro-adhesive signals from TSP1 in the extracellular matrix. Both the IGF1 receptor and CD98 are components of this regulatory complex in breast carcinoma cells, but the mechanisms of their actions also remain to be defined.

Although several signaling pathways have been identified that regulate integrin activity by "inside-out" signaling (39), the mechanisms for regulating activation states of specific integrins remain poorly understood. In contrast to $\alpha_v\beta_3$ integrin, the $\alpha_3\beta_1$ integrin in breast carcinoma cells is not activated by engagement of CD47 by the TSP1 "VVM" peptides or by protein kinase C activation. Rather, inhibition of Ser/Thr kinase activity, but not Tyr kinase activity, increases β_1-mediated adhesive activity of MDA-MB-435 cells for TSP1. Conversely, phorbol ester activation of protein kinase C increased adhesion via $\alpha_v\beta_3$ but not $\alpha_3\beta_1$ integrin. Thus, activation of individual integrins in MDA-MB-435 cells can be differentially regulated.

We have identified the IGF1 receptor as a specific regulator of $\alpha_3\beta_1$-mediated interactions with TSP1. The insulin and IGF1 receptors were reported to be physically associated with the $\alpha_v\beta_3$ integrin but not with β_1 integrins in fibroblasts (40). The $\alpha_v\beta_3$ integrin also co-immunoprecipitated with insulin receptor substrate-1 (41). Engagement of $\alpha_v\beta_3$ integrin by vitronectin but not $\alpha_2\beta_1$ integrin by collagen increased mitogenic signaling through the insulin receptor (40, 41). Thus, the specific activation of $\alpha_3\beta_1$-mediated spreading and chemotaxis to TSP1 by insulin or IGF1 was unexpected. We observed a stronger response for stimulating adhesion to TSP1 than to collagen or laminin, suggesting that the regulation of avidity by the IGF1 receptor is specific for the $\alpha_3\beta_1$ integrin. Other growth factors that utilize tyrosine kinase receptors including FGF2 and EGF did not activate this integrin. We therefore predict that specific coupling of $\alpha_3\beta_1$ activation to IGF1 receptor signaling, rather than a general phosphorylation signal, mediates rapid activation of the TSP1 binding integrin in breast carcinoma cells. The mechanism for this specific signaling remains to be determined.

CD98 was recently identified as an activator of β_1 integrins by its ability to overcome Tac-β1 suppression of β_1 integrin function (36, 42). Our data demonstrate that clustering of CD98 can also increase $\alpha_3\beta_1$-mediated TSP1 interactions. This may simply result from clustering of the CD98-associated $\alpha_3\beta_1$ integrin, which increases the avidity for cell adhesion to a surface coated with TSP1, or it may require specific signal transduction from CD98. Regulation of CD98 levels is probably responsible for the serum-induced increase in adhesion to TSP1, since serum increases CD98 surface expression in MDA-MB-435 cells. The insulin and IGF1-induced stimulation of TSP1 spreading and chemotaxis cannot be explained by regulation of CD98 levels, however, since IGF1 down-regulates CD98 in these cells.

Only a small fraction of the $\alpha_3\beta_1$ integrin on MDA-MB-231 and MCF-7 cells is constitutively active to mediate adhesion to TSP1. The inactive integrin appears to be on the cell surface, since it can be rapidly activated by the TS2/16 antibody or by IGF1 receptor ligands. The low basal activity of this integrin could result from absence of an activator or expression of an inhibitor in MDA-MB-231 and MCF-7 cells. Several factors that suppress integrin function have been identified, including Ha-Ras (43), integrin-linked kinase, and protein kinase C (39). Additional proteins are known to associate with the $\alpha_3\beta_1$ integrin, including some members of the TM4SF family and EMMPRIN (44, 45), but their roles in regulating function are unknown. In MDA-MB-231 cells, suppression of $\alpha_3\beta_1$ appears to be an active process that can be disrupted by PT. Thus, a heterotrimeric G-protein signaling pathway appears to maintain MDA-MB-231 cells in an inactive state. This inhibitory pathway may also be specific for the $\alpha_3\beta_1$ integrin in MDA-MB-231 cells, because unstimulated MDA-MB-231 cells can spread on type I collagen using $\alpha_2\beta_1$ integrin. Unstimulated MDA-MB-435 cells show the opposite phenotype, with better $\alpha_3\beta_1$-dependent adhesion to TSP1 than $\alpha_2\beta_1$-dependent adhesion to collagen. The differential modulation of TSP1 interactions with these two cell lines by PT as well as the calcium ionophores demonstrates that regulation of $\alpha_3\beta_1$ activity for TSP1 may differ even between two cell lines derived from the same type of human cancer.

TSP1 has diverse effects on breast carcinoma cell behavior, altering their adhesion, motility, proliferation, protease expression, and invasion. These cellular responses result in alterations of their *in vivo* tumorigenic, angiogenic, and metastatic potentials (reviewed in Ref. 4). We have defined specific roles for the $\alpha_3\beta_1$ integrin in spreading, induction of filopodia, and chemotactic responses to TSP1. In other cell types, the low density lipoprotein receptor-related protein has been assigned a role in internalization of TSP1 (46), and CD36 has been shown to play an essential role in angiogenesis inhibition (47). The receptors that mediate many responses to TSP1 remain to be defined. These responses may require coordinated signaling through two or more TSP1 receptors. Defining the role of IGF1 and CD98 in regulating β_1 integrin interactions with TSP1 provides our first insight into a breast carcinoma TSP1 receptor that can be turned on or off in response to known environmental stimuli. The ability to regulate the activity of this TSP1 receptor will facilitate analysis of the signals resulting from this interaction.

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Identification of an $\alpha_3\beta_1$ Integrin Recognition Sequence in Thrombospondin-1*

(Received for publication, January 25, 1999, and in revised form, April 21, 1999)

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A synthetic peptide containing amino acid residues 190–201 of thrombospondin-1 (TSP1) promoted adhesion of MDA-MB-435 breast carcinoma cells when immobilized and inhibited adhesion of the same cells to TSP1 when added in solution. Adhesion to this peptide was enhanced by a β_1 integrin-activating antibody, Mn^{2+}, and insulin-like growth factor I and was inhibited by an $\alpha_3\beta_1$ integrin function-blocking antibody. The soluble peptide inhibited adhesion of cells to the immobilized TSP1 peptide or spreading on intact TSP1 but at the same concentrations did not inhibit attachment or spreading on type IV collagen or fibronectin. Substitution of several residues in the TSP1 peptide with Ala residues abolished or diminished the inhibitory activity of the peptide in solution, but only substitution of Arg-198 completely inactivated the adhesive activity of the immobilized peptide. The essential residues for activity of the peptide as a soluble inhibitor are Asn-196, Val-197, and Arg-198, but flanking residues enhance the inhibitory activity of this core sequence, either by altering the conformation of the active sequence or by interacting with the integrin. This functional sequence is conserved in all known mammalian TSP1 sequences and in TSP1 from *Xenopus laevis*. The TSP1 peptide also inhibited adhesion of MDA-MB-435 cells to the laminin-1 peptide GD6, which contains a potential integrin-recognition sequence Asn-Leu-Arg and is derived from a similar position in a pentraxin module. Adhesion studies using recombinant TSP1 fragments also localized β1 integrin-dependent adhesion to residues 175–242 of this region, which contain the active sequence.

Expression of the $\alpha_3\beta_1$ integrin is essential for normal development in the kidney and lungs (1). Targeted mutation of the murine α_3 *integrin* gene resulted in abnormal branching morphogenesis of kidney capillary loops and lung bronchi. Based on antibody inhibition, this integrin may also be important for branching morphogenesis in mammary epithelia (2). In addition to its essential roles in normal development, the $\alpha_3\beta_1$ integrin may play important roles in disease processes, such as cancer. Loss of integrin α_3 subunit expression is a negative prognostic factor in lung adenocarcinoma (3). Conversely, overexpression of $\alpha_3\beta_1$ integrin in a human rhabdomyosarcoma line suppressed tumor formation in mouse xenografts (4).

The $\alpha_3\beta_1$ integrin has been reported to recognize several extracellular matrix ligands, including some laminins, type IV collagen, fibronectin, thrombospondin-1, and entactin/nidogen (5–8). Although short peptide recognition motifs have been identified in ligands for some integrins (reviewed in Ref. 9), previous attempts to define recognition sequences for binding of matrix ligands to the $\alpha_3\beta_1$ integrin have produced conflicting results. High affinity binding of recombinant soluble $\alpha_3\beta_1$ could be detected only to laminin-5 (10), so binding to other matrix ligands may be of relatively low affinity. Under specific conditions, this integrin can recognize the common integrin binding sequence RGD in fibronectin (6). However, recombinant entactin with the RGD sequence deleted (11) and synthetic peptides from laminin-1 and type IV collagen that lack the RGD motif (12, 13) also bound specifically to the $\alpha_3\beta_1$ integrin. Laminin peptide GD6 (KQNCLSSRASFRGCVRNLRLSR) and the type IV collagen peptide affinity purified $\alpha_3\beta_1$ integrin from cell extracts when immobilized on agarose beads (12, 13), but the active peptides from these two proteins share no apparent sequence homology. These data, combined with the evidence that RGD-dependent and RGD-independent adhesion are differentially regulated in $\alpha_3\beta_1$ integrin (6), have led to the proposal that the $\alpha_3\beta_1$ integrin uses distinct mechanisms to interact with each of its ligands and that no conserved binding motif may exist (6).

We recently found that $\alpha_3\beta_1$ is the major TSP1[1]-binding integrin on several human breast carcinoma cell lines (14). We have further examined this interaction and report the identification of a peptide sequence from TSP1 that supports $\alpha_3\beta_1$-dependent adhesion and chemotaxis and is a potent inhibitor of adhesion to TSP1.

EXPERIMENTAL PROCEDURES

Proteins and Peptides—Calcium replete TSP1 was purified from human platelets (15). Synthetic peptides containing TSP1 sequences were prepared as described previously (16–21). Recombinant fragments (provided by Dr. Tikva Vogel) and GST fusion proteins expressing fragments of TSP1 (provided by Dr. Jack Lawler, Harvard University) were prepared as described previously (22, 23). Bovine type I collagen and murine Type IV collagen were obtained from Becton Dickinson Labware division, and human vitronectin was from Sigma. Fibronectin was purified from human plasma (National Institutes of Health Blood Bank) as described (24). Murine laminin-1 purified from the EHS tumor was provided by Dr. Sadie Aznavoorian (NCI, National Institutes of Health). Recombinant human insulin-like growth factor-1 (IGF1) was from Bachem.

Adhesion Assays—Adhesion was measured on polystyrene or glass substrates coated with peptides or proteins as described previously (16). Inhibition assays were performed using the following function-blocking antibodies: 6D7 ($\alpha_2\beta_1$), P1B5 (Life Technologies, Inc., $\alpha_3\beta_1$), 407279 (Calbiochem, $\alpha_4\beta_1$), and P1D6 (Life Technologies, Inc., $\alpha_5\beta_1$). The β_1 integrin-activating antibody TS2/16 (25) was prepared from the hybridoma obtained from the American Type Culture Collection. Immunofluorescence analysis of cell adhesion was performed as described previously, using BODIPY TR-X phallacidin (Molecular Probes, Inc., Eugene, OR) to visualize F-actin or using murine primary antibodies

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[1] The abbreviations used are: TSP, human thrombospondin; GST, glutathione *S*-transferase; IGF1, insulin-like growth factor-1.

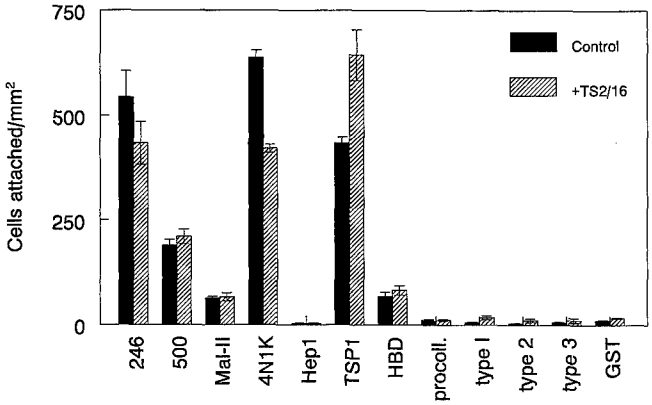

FIG. 1. **Adhesion of MDA-MB-435 breast carcinoma cells to recombinant TSP1 fragments and synthetic TSP1 peptides.** Adhesion to synthetic TSP1 peptides adsorbed at 10 μM (*246,* KRFKQDGGWSHWSPWSS; *500,* NGVQYRNC; *Mal II,* SPWSSCSVTCGDGVITRIR; *4N1K,* KRFYVVMWKK; *HepI,* ELTGAARKGSGRRLVKGPD), TSP1 (0.11 μM), recombinant 18-kDa heparin-binding domain (*HBD*) (2.7 μM), or GST fusion proteins expressing the TSP1 procollagen domain (*procoll.*), type 1, 2, or 3 repeats, or GST alone (2 μM) was measured in the absence (*solid bars*) or presence (*striped bars*) of 20 μg/ml of the β_1 integrin-activating antibody TS2/16. Results (mean ± S.D) are presented for a representative experiment performed in triplicate.

followed by BODIPY FL anti-mouse IgG to localize integrins, vinculin (Sigma), or focal adhesion kinase (clone 77, Transduction Laboratories) (26).

Motility Assays—Chemotaxis of MDA-MB-435 cells to TSP1 peptides was measured in modified Boyden chambers using polylysine-coated 8 μm polycarbonate filters as described previously for intact TSP1 (14).

Multiple Sequence Alignment—Protein sequences were compared using MACAW software (National Center for Biotechnology Information, National Library of Medicine, version 2.0.5) by the segment pair overlap and Gibbs sampler methods (27, 28).

RESULTS

In initial attempts to localize the region of TSP1 recognized by the $\alpha_3\beta_1$ integrin, we tested approximately 85% of the TSP1 sequence in the form of synthetic peptides or GST or T7 fusion proteins for promotion of β_1 integrin-dependent adhesion of MDA-MB-435 cells (Fig. 1). Among the recombinant fragments tested, only an 18-kDa fragment of the N-terminal heparin-binding domain had significant adhesive activity, although the recombinant type I repeats had adhesive activity for MDA-MB-435 cells in some experiments (results not shown). A recombinant GST fusion of the type 3 repeats of TSP1 including the RGD sequence had minimal adhesive activity for MDA-MB-435 cells (Fig. 1), in contrast to human melanoma cells, which avidly attached on substrates coated with the same concentrations of this fragment (26). The β_1 integrin-activating antibody TS2/16 did not enhance cell attachment to any of these recombinant fragments but reproducibly stimulated attachment on intact TSP1 (Fig. 1). Synthetic heparin-binding peptides from the type 1 repeats (peptide 246) (16) and the CD47-binding peptide 4N1K (21) also promoted adhesion, but TS2/16 did not enhance adhesion of MDA-MB-435 cells to these peptides. CD36-binding peptides from the procollagen domain (peptide 500) or the type 1 repeats (Mal-II) (29) had weaker adhesive activities and were also insensitive to TS2/16. The focal adhesion disrupting peptide Hep1 from the N-terminal domain of TSP1 (20) did not promote MDA-MB-435 cell adhesion. Although these experiments did not detect a β_1 integrin-dependent adhesive sequence in TSP1, the possibility remains that these regions of TSP1 contain a conformation-dependent recognition motif that is inactive in the recombinant fusion proteins due to misfolding.

A multiple alignment search using MACAW software was

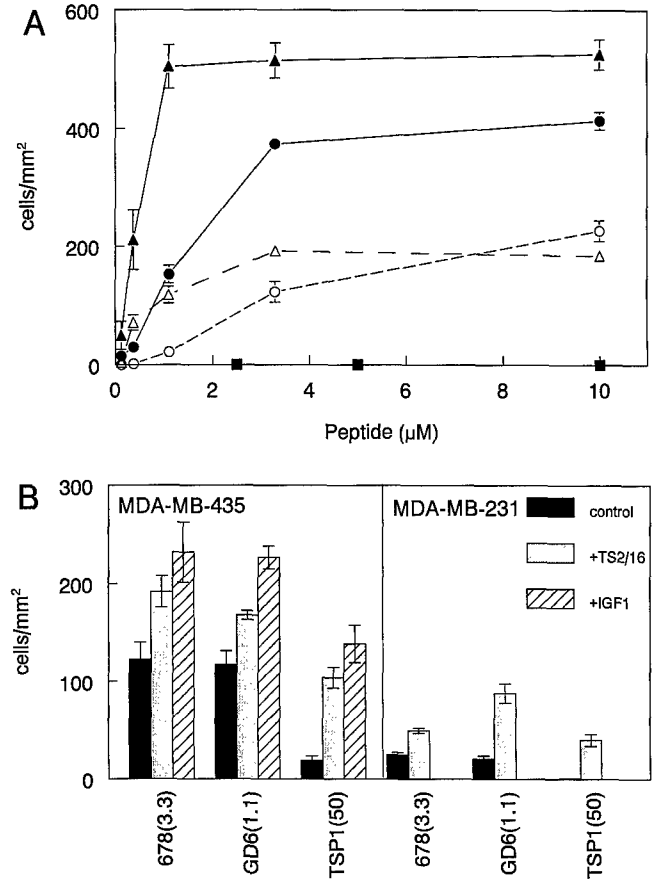

FIG. 2. **MDA-MB-435 adhesion to TSP1 peptides and laminin-1 peptide GD6.** *A,* MDA-MB-435 breast carcinoma cell attachment (*closed symbols*) and spreading (*open symbols*) was determined on polystyrene substrates coated with the indicated concentrations of TSP1 peptide 678 (FQGVLQNVRFVF) (*circles*), TSP1 peptide 701 (TPGQVRTLWHDP) (*squares*), or the murine laminin-1 peptide GD6 (KQNCLSSRASFRGCVRNLRLSR) (*triangles*). Results are presented as mean ± S.D. (n = 3). *B,* spreading of MDA-MB-435 or MDA-MB-231 cells on substrates coated with 3.3 μM TSP1 peptide 678, 1.1 μM laminin-1 peptide GD6, or 50 μg/ml TSP1 was determined using untreated cells (*black bars*) or cells treated with 5 μg/ml of the β_1-activating antibody TS2/16 (*gray bars*) or 3 nM IGF1 (*striped bars*) (MDA-MB-435 cells only); mean ± S.D. (n = 3).

used to identify TSP1 sequences that might be related to the $\alpha_3\beta_1$ integrin-binding GD6 peptide derived from the A chain of murine laminin-1 (12), which strongly promoted MDA-MB-435 cell adhesion (Fig. 2*A*). This search identified four TSP1 sequences related to the laminin peptide (Table I). The single peptide identified by the Gibbs sampler method, derived from the C-terminal domain of TSP1 (residues 1059–1077), did not support adhesion or inhibit adhesion of MDA-MB-435 cells to TSP1 or other $\alpha_3\beta_1$ integrin ligands (Fig. 2*A* and results not shown). Because a synthetic peptide containing the last 12 residues of peptide GD6 (peptide 679, Table I) had similar activity to the intact peptide (see below), we did not test the two peptides identified by segment pair overlap that aligned outside this sequence. Both of these peptides were derived from regions of the type 1 (residues 392–405) or type 2 (residues 598–608) repeat sequences that did not support $\alpha_3\beta_1$-dependent adhesion when expressed as GST fusion proteins (Fig. 1).

The remaining sequence is from a region of the N-terminal domain of TSP1 (residues 188–199) that was not covered by the recombinant fragments tested in Fig. 1 and conserves most of the hydrophilic residues in the laminin-1 GD6 peptide that could mediate protein-protein interactions. This sequence also overlaps with a region identified in a screen of N-terminal

TABLE I
TSP1 sequences related to murine laminin-1 peptide GD6

The amino acid sequences for human and murine TSP1 and laminin-1 peptide GD6 were compared by multiple alignment using MACAW.

Peptide origin	Sequence	MP score *vs.* GD6	*p* value
Laminin GD6	KQNCLSSRASFRGCVRNLRLSR		
Laminin p679	FRGCVRNLRLSR		
TSP1 (598–608)	NCLPCPPRFTG	42.0[a]	5.9×10^{-8}
TSP1 (188–199)	DNFQGVLQNVRF	39.0[a]	5.9×10^{-7}
TSP1 (392–405)	NNRCEGSSVQTRTC	35.0[a]	4.5×10^{-4}
TSP1 (1059–1077)	RNALWHTGNTPGQVRTLWH	43.3[b]	2.1×10^{-8}

[a] Alignment scores were determined by segment pair overlap.
[b] Alignment by Gibbs sampler method.

TSP1 peptides as having heparin-independent adhesive activity (30). A synthetic peptide containing this TSP1 sequence (peptide 678) had strong adhesive activity for MDA-MB-435 cells (Fig. 2*A*). Spreading of two breast carcinoma cell lines on this peptide, laminin peptide GD6, and TSP1 was enhanced in the presence of the β_1 integrin-activating antibody TS2/16 (Fig. 2*B*). We previously found that IGF1 strongly stimulated β_1 integrin-mediated adhesion to TSP1 (14). IGF1 similarly stimulated spreading of MDA-MB-435 cells on the TSP1 peptide 678 and to the laminin peptide GD6 (Fig. 2*B*).

The TSP1 peptide 678 strongly inhibited spreading of MDA-MB-435 cells on TSP1 and murine EHS tumor-derived laminin-1/entactin but did not inhibit spreading of the same cells on the $\alpha_5\beta_1$ integrin ligand fibronectin or on type IV collagen (Fig. 3*A*). The TSP1 peptide in solution strongly inhibited MDA-MB-435 cell attachment to itself and to GD6 (Fig. 3*B*), a known $\alpha_3\beta_1$ integrin-binding peptide from murine laminin-1 (12). In contrast, the laminin peptide was a relatively weak inhibitor of adhesion to either peptide or TSP1 when tested in solution (IC_{50} = 700 μM, data not shown).

The TSP1 peptide 678 sequence was not in the recombinant N-terminal fragment tested in Fig. 1, but the previously reported 28-kDa N-terminal fragment of TSP1 contains this sequence (22). Adhesion assays using MDA-MB-231 (Fig. 3*C*) and MDA-MB-435 breast carcinoma cell lines (data not shown) verified that the larger fragment, expressing residues 1–242, contains a β_1 integrin-dependent adhesion sequence that is not present in residues 1–174. Adhesion to the longer fragment was stimulated by the β_1-activating antibody TS2/16 and inhibited by peptide 678 (Fig. 3*C*). Therefore, a β_1 integrin-binding site is present in residues 175–242 of TSP1 and is functional when expressed as a recombinant protein.

To verify that the TSP1 peptide 678 contains an $\alpha_3\beta_1$ integrin recognition sequence, integrin α-subunit antibodies were tested for blocking adhesion to the peptide (Fig. 4). The α_3-specific blocking antibody P1B5, which we have shown to inhibit adhesion of the same cells to intact TSP1 (14), partially inhibited adhesion of MDA-MB-435 cells on peptide 678 and completely reversed the enhancement of MDA-MB-435 cell adhesion to the same peptide stimulated by the β_1 integrin-activating antibody TS2/16. In a further control experiment, the $\alpha_2\beta_1$-blocking antibody 6D7 inhibited adhesion of MDA-MB-435 cells to type I collagen but not to peptide 678 (Fig. 4*B*). Function-blocking antibodies for $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins also had no effect on adhesion to peptide 678 (data not shown). Therefore, the peptide does not support adhesion mediated by $\alpha_4\beta_1$ or $\alpha_5\beta_1$ integrins or inhibit adhesion to other integrin ligands.

Divalent cation dependence is also characteristic for binding of integrin ligands. Although Mn^{2+} but not Ca^{2+} induced the expected increase in MDA-MB-435 cell spreading on TSP1 peptide 678 and intact TSP1 (Fig. 4*C*), addition of EDTA only minimally inhibited basal spreading on peptide 678. EDTA completely inhibited the spreading on TSP1 observed in medium containing Mg^{2+} as the sole divalent cation, although it did not inhibit cell attachment on TSP1 (Fig. 4*C* and results not shown). This residual adhesion probably results from the significant contribution of proteoglycans to adhesion of MDA-MB-435 cells on TSP1 (14). Spreading on peptide 678 with Mg^{2+} as the divalent cation became partially sensitive to EDTA, however, in the presence of the β_1-activating antibody TS2/16. Addition of Mn^{2+} further stimulated spreading on peptide 678

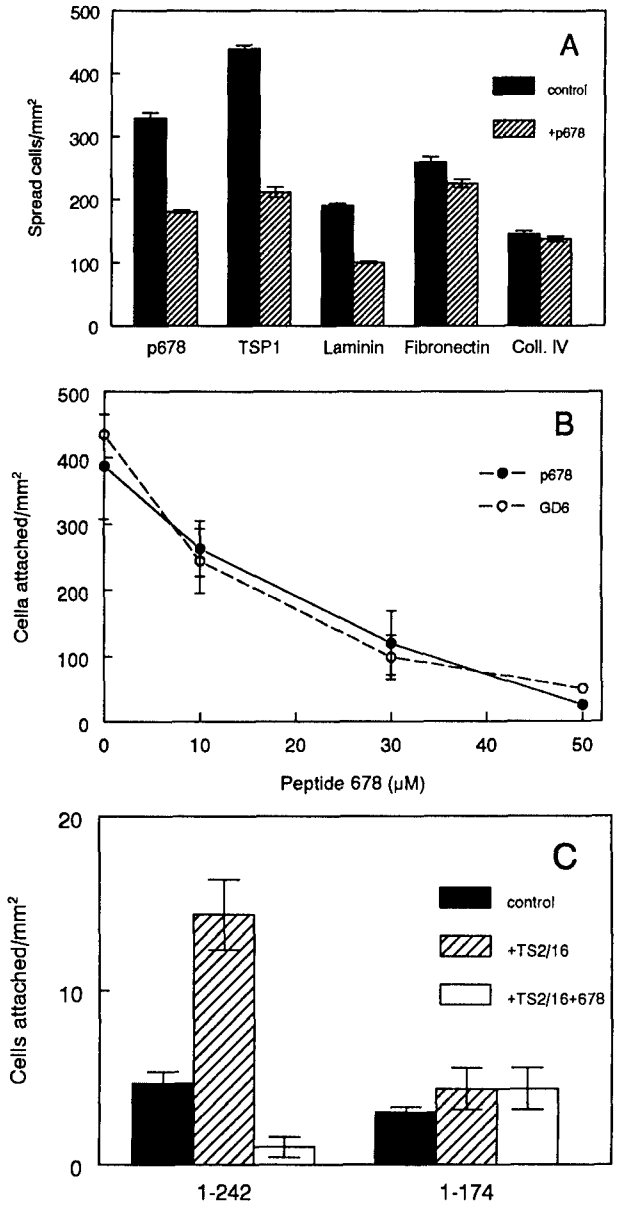

FIG. 3. **Inhibition of breast carcinoma cell spreading on matrix proteins by peptide 678.** *A*, MDA-MB-435 cell spreading was determined in the absence (*solid bars*) or presence (*striped bars*) of 10 μM TSP1 peptide 678 on substrates coated with 10 μM peptide 678, 40 μg/ml TSP1, 10 μg/ml murine laminin-1, 10 μg/ml human plasma fibronectin, or 10 μg/ml type IV collagen. Cell spreading is presented as mean ± S.D. (n = 3). *B*, inhibition of MDA-MB-435 cell adhesion to surfaces coated with 10 μM peptide 678 (●) or laminin peptide GD6 (○) was measured in the presence of the indicated concentrations of peptide 678 added in solution. *C*, adhesion of MDA-MB-231 cells to the indicated recombinant TSP1 fragments was measured in RPMI medium containing 0.1% bovine serum albumin (*black bars*) or the same medium containing 5 μg/ml of the β_1-activating antibody TS2/16 (*striped bars*) or TS2/16 plus 20 μM peptide 678 (*open bars*). Cell attachment is presented as mean ± S.D. for triplicate determinations.

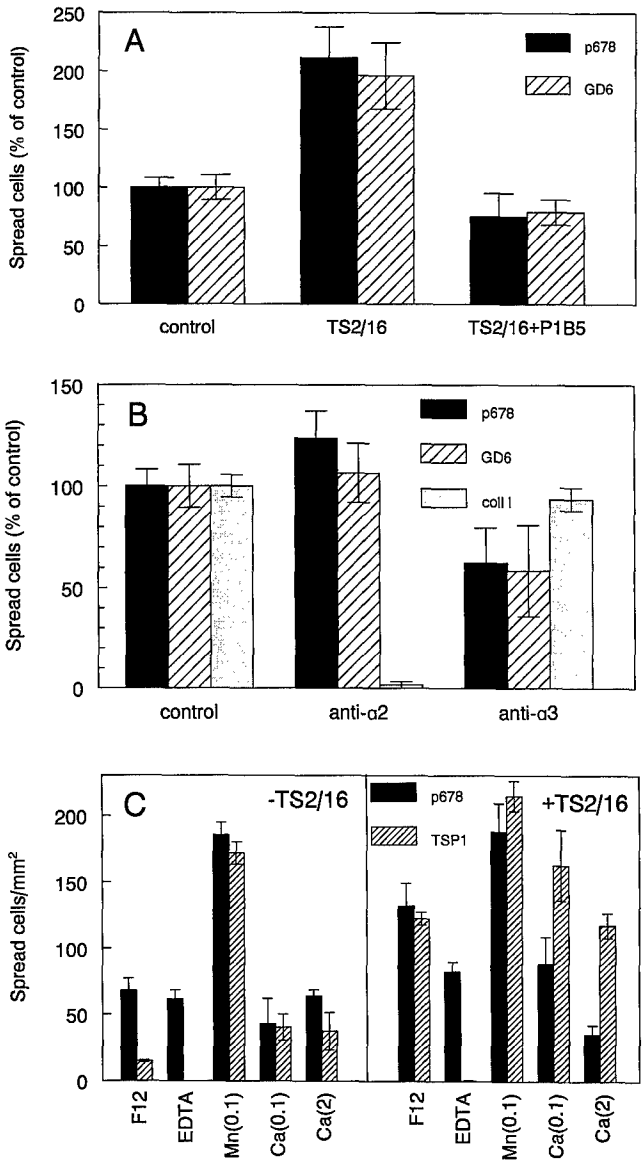

FIG. 4. **The $\alpha_3\beta_1$ integrin mediates adhesion to TSP1 peptide 678 and laminin-1 peptide GD6.** *A*, MDA-MB-435 cell spreading on TSP1 peptide 678 (*solid bars*) or laminin-1 peptide GD6 (*striped bars*) was determined with no additions (control), in the presence of 5 μg/ml of β_1-integrin antibody TS2/16, or in the presence of 5 μg/ml each of antibody TS2/16 and the $\alpha_3\beta_1$-blocking antibody P1B5. Results are normalized to the control and are presented as mean ± S.D. (n = 3). *B*, MDA-MB-435 cell spreading on substrates coated with 10 μM TSP1 peptide 678 (*black bars*), 5 μM laminin-1 peptide GD6 (*striped bars*), or 5 μg/ml type I collagen (*gray bars*) was determined in the presence of 5 μg/ml of the $\alpha_2\beta_1$-blocking antibody 6D7 (anti-α_2) or the $\alpha_3\beta_1$-blocking antibody P1B5 (anti-α_3). Results are normalized to untreated controls and presented as mean ± S.D. (n = 3). *C*, divalent cation dependence for adhesion on TSP1 peptide 678 and intact TSP1. MDA-MB-435 cells were suspended in calcium-free Ham's F-12(K) medium containing 2 mM magnesium and the indicated concentrations of divalent cations or 2.5 mM EDTA. Cell spreading on substrates coated with 5 μM peptide 678 (*solid bars*) or 40 μg/ml TSP1 (*striped bars*) was determined in the absence or presence of 5 μg/ml of the β_1 integrin-activating antibody TS2/16.

and intact TSP1 in the presence of TS2/16, but addition of Ca^{2+} produced a dose-dependent inhibition of spreading on both substrates. Specific inhibition by Ca^{2+} is consistent with previous data for the $\alpha_3\beta_1$ integrin (31). These results suggest that integrin binding to peptide 678 is partially independent of divalent cations, but MDA-MB-435 cell spreading on this peptide may involve both $\alpha_3\beta_1$ integrin binding and divalent cation-independent interactions with another cell surface molecule.

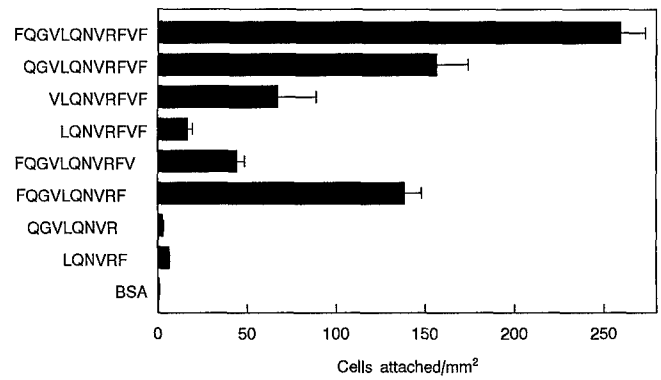

FIG. 5. **Determination of the minimal active TSP1 sequence to promote breast carcinoma cell adhesion.** MDA-MB-435 cell adhesion was determined to polystyrene coated with 10 μM of the indicated peptides or with bovine serum albumin (*BSA*). Cell attachment is presented as the mean ± S.D. for triplicate determinations.

Truncated peptides that contained portions of peptide 678 were synthesized to identify essential residues (Fig. 5). Truncation of the N-terminal Phe or the C-terminal Val-Phe only moderately decreased adhesive activity, but further truncations from either end of the peptide greatly diminished its activity. Inhibition assays confirmed that the loss of adhesive activity reflected loss of integrin binding rather than loss of ability to adsorb on the substrate (Table II). As found in the direct adhesion assays, peptides without the N-terminal Phe or the C-terminal Val-Phe retained significant inhibitory activities, but all shorter peptides were weak inhibitors or inactive. These results imply that the integrin recognizes an extended sequence, but this approach could not discriminate conformational effects of flanking sequences from a direct contribution to integrin binding.

To better define those residues involved in $\alpha_3\beta_1$ integrin binding, we systematically substituted Ala residues into the peptide 678 and tested each for adhesive activity (Fig. 6). Based on the complete loss of adhesion activity for MDA-MB-435 cells following its substitution, only Arg-198 was essential for adhesive activity of peptide 678 (Fig. 6). Replacement of Arg-198 with a His also dramatically reduced adhesive activity. Ala substitutions at several other positions significantly decreased adhesive activity, except for the two N-terminal residues, which only slightly decreased adhesive activity.

Although only the Arg residue was essential for direct adhesion, substitution of several additional residues with Ala markedly decreased or abolished inhibitory activity of the respective soluble peptides in solution to block $\alpha_3\beta_1$-dependent adhesion to immobilized peptide 678 (Table III). These experiments showed that Arg-198, Val-197, and Asn-196 are essential for inhibitory activity of the peptides in solution. Substitution of Phe-199 and Phe-201 decreased the inhibitory activities of the respective peptides 5–8-fold, indicating that these flanking residues also contribute to activity of the peptides in solution. In contrast, peptides with Ala substitutions at four of the six N-terminal residues in this sequence had inhibitory activities equivalent to that of the native TSP1 sequence. Therefore, NVR is the essential sequence for binding to the $\alpha_3\beta_1$ integrin, but flanking residues may be necessary for inducing the proper conformation of this minimal sequence in peptide 678.

The specificity for an Arg residue at position 198 was further examined using conservative amino acid substitutions (Table III). Substitution with Lys decreased activity approximately 2-fold, whereas substitution with Gln, to retain hydrogen-bonding ability while removing the positive charge, abolished the inhibitory activity. A His substitution showed intermediate activity, indicating that a positive charge rather than a large

TABLE II
Inhibition of MDA-MB-435 cell adhesion to immobilized peptide 678 by fragments of the active peptide

Mean doses to achieve 50% inhibition of control adhesion (IC_{50}) to polystyrene coated with 5 μM peptide 678 were determined from at least three independent experiments, each performed in triplicate. Peptides were tested at up to 300 μM or to the solubility limit for each peptide, where lower limits for inhibitory activity are indicated.

Peptide	Sequence	IC_{50}
		μM
678	FQGVLQNVRFVF (TSP1)	3.5
682	FQGVLQNVRF	6
683	QGVLQNVR	>300
685	QGVLQNVRFVF	24
688	VLQNVRFVF	>100
684	LQNVRFVF	300
681	ac-LQNVRF-am	700

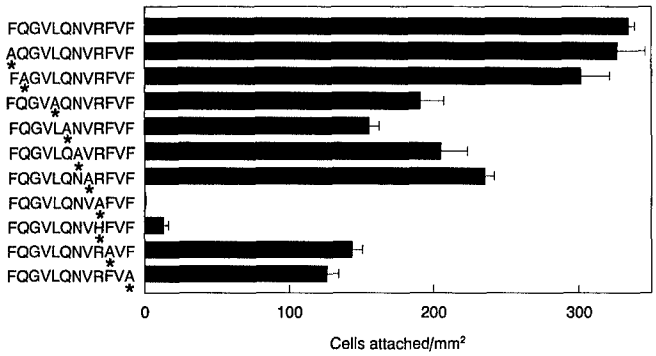

FIG. 6. **Effect of systematic substitution of Ala residues on adhesive activities of the TSP1 sequence 190–201 for breast carcinoma cells.** Cell attachment was determined to substrates coated with each peptide at 10 μM and is presented as mean ± S.D. (n = 3). Residues substituted in the native TSP1 sequence are indicated with an *asterisk*.

TABLE III
Mapping of essential residues for inhibition of MDA-MB-435 cell adhesion to immobilized peptide 678

Mean doses to achieve 50% inhibition of control adhesion to 5 μM peptide 678 (IC_{50}) were determined from at least three independent experiments, each performed in triplicate. Residues substituted in the native TSP1 sequence are underlined.

Peptide	Sequence	IC_{50}
		μM
678	FQGVLQNVRFVF (TSP1)	3.5
697	AQGVLQNVRFVF	5
696	FAGVLQNVRFVF	1.8
695	FQGVAQNVRFVF	5
687	FQGVLANVRFVF	3
686	FQGVLQAVRFVF	>300
691	FQGVLQNARFVF	>300
690	FQGVLQNVAFVF	>300
702	FQGVLQNVKFVF	6
694	FQGVLQNVHFVF	54
703	FQGVLQNVQFVF	>100
692	FQGVLQNVRAVF	18
693	FQGVLQNVRFVA	27

side chain with hydrogen bonding ability is required at this position.

The active peptides strongly promoted formation of filopodia in MDA-MB-435 cells (Fig. 7*A*) similar to those induced by attachment on intact TSP1 (14). Addition of IGF1 enhanced spreading and increased formation of lamellipodia on the same peptide (Fig. 7*B*). Phallacidin staining demonstrated organization of F-actin at the cell periphery but no organization of stress fibers across the cell body (Fig. 7*C*). Using antibodies recognizing vinculin (Fig. 7*D*) and focal adhesion kinase (data not shown) as markers of focal adhesion formation, we could not

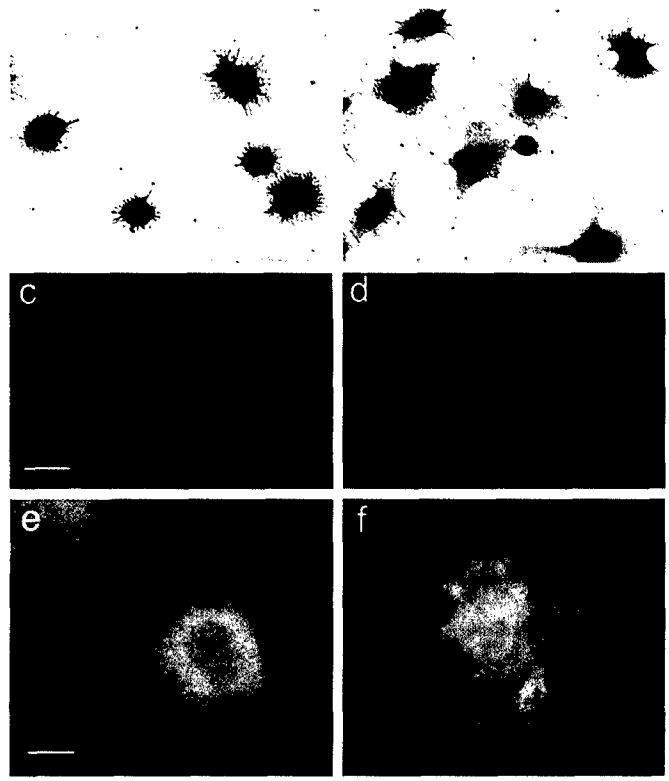

FIG. 7. **Morphology of MDA-MB-435 cells attaching on TSP1 peptide 678.** *a*, direct adhesion on TSP1 peptide 678 stimulates formation of filopodia (*bar*, 50 μm). *b*, IGF1 stimulates increased spreading with formation of lamellipodia. *c*, staining of F-actin using BODIPY TR-X phallacidin (*bar*, 20 μm). *d*, double labeling of the field in *c* with anti-vinculin antibody. *e*, immunolocalization of β_1 integrin subunits in cells attached on peptide 678 using antibody TS2/16 (*bar*, 10 μm). *f*, immunolocalization of α_3 integrin subunits using antibody P1B5.

detect any induction of focal adhesions in MDA-MB-435 cells attaching on these peptides, although the same markers showed typical focal adhesion staining patterns in the cells when attaching on vitronectin or fibronectin substrates (results not shown). Staining for the $\alpha_3\beta_1$ integrin was punctate and prominently localized in filopodia extended by MDA-MB-435 cells on immobilized peptide 678 (Fig. 7*F*), whereas total β_1 integrin staining was more diffuse and concentrated over the cell body.

TSP1 stimulates chemotaxis of MDA-MB-435 cells, and this response is inhibited by the $\alpha_3\beta_1$-blocking antibody P1B5 (14). Peptide 678 also stimulated chemotaxis of MDA-MB-435 cells (Fig. 8). Chemotaxis to peptide 678 was dose-dependent with a maximal response at 10 μM (Fig. 8*A*). This response was specific in that peptide 690 was inactive. In agreement with the observations that IGF1 stimulated β_1 integrin-dependent chemotaxis of MDA-MB-435 cells to TSP1 (14) and adhesion of the same cells to peptide 678 (Figs. 2 and 7), the chemotactic response of MDA-MB-435 cells to peptide 678, but not to peptide 690, was increased in the presence of IGF1 (Fig. 8*B*).

DISCUSSION

Based on examination of synthetic peptides and recombinant fragments representing approximately 90% of the TSP1 sequence, only the sequence FQGVLQNVRFVF from the N-terminal domain exhibited activities that are expected for an $\alpha_3\beta_1$ integrin binding sequence in TSP1. A recombinant fragment of TSP1 containing this sequence also promoted β_1 integrin-dependent adhesion. In solution, this peptide specifically inhibited adhesion to TSP1 but not to ligands recognized by other integrins. Adhesion to this peptide and to TSP1 was stimulated by IGF1 receptor ligands that stimulate integrin-dependent

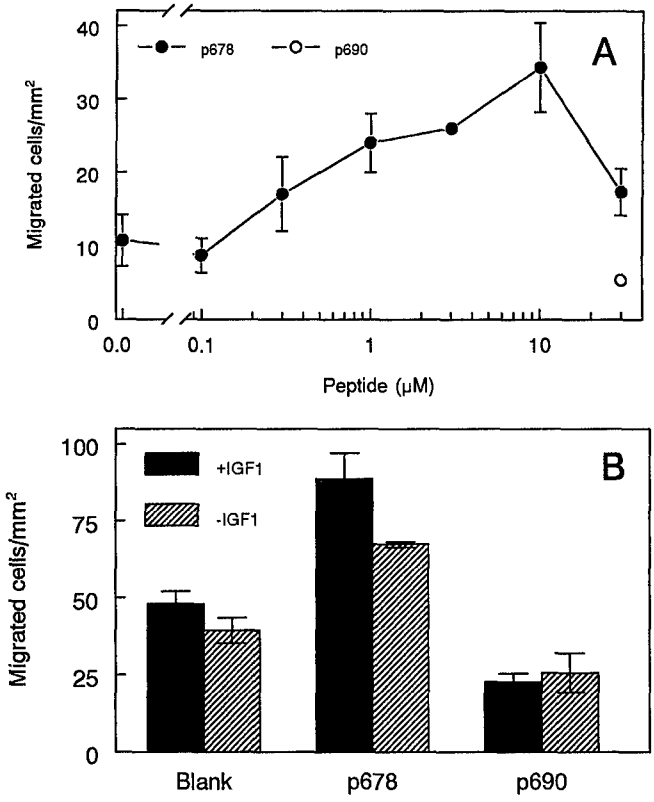

FIG. 8. **TSP1 peptide 678 promotes breast carcinoma cell chemotaxis.** *A*, dose dependence for stimulation of MDA-MB-435 cell motility by peptide 678 added to the lower well of a modified Boyden chamber. Cells that migrated to the lower surface of an 8-μm-pore polycarbonate filter were quantified microscopically after 7 h; the data shown are mean ± S.D. (n = 3) for a representative experiment. *B*, MDA-MB-435 cell chemotaxis was measured to medium alone (*Blank*) or to 10 μM TSP1 peptide 678 or 10 μM inactive analog peptide 690 added to the lower chamber. Chemotaxis of untreated cells (*striped bars*) or cells treated with 10 nM IGF1 in the upper chamber (*solid bars*) was determined after 7 h and is presented as mean ± S.D. (n = 3).

adhesion to intact TSP1, by Mn^{2+}, and by a β_1 integrin-activating antibody and partially inhibited by an $\alpha_3\beta_1$ function-blocking antibody. Based on systematic amino acid substitutions in the active sequence, NVR appears to be the essential core sequence in this TSP1 peptide for recognition by the $\alpha_3\beta_1$ integrin.

Adhesive activities of the immobilized peptides imply that only Arg-198 may directly participate in this interaction, although the partial resistance to inhibition by an $\alpha_3\beta_1$ integrin antibody and EDTA suggest that the peptides with Arg may also support adhesion independent of integrin binding. The context surrounding the Arg is important, however, because other peptides with similar sequences (such as peptide 701, with a QVRT sequence) had no activity, and Ala substitutions of the flanking residues in peptide 678 eliminated or markedly decreased its inhibitory activity in solution. The essential amino acid residues are completely conserved in human, murine, bovine, and *Xenopus* TSP1, but in chicken TSP1, a His replaces the Arg. A similar motif is found in murine and human TSP2, with a His residue replacing the Arg. As a free peptide, the TSP1 sequence with a His substitution was much less active, so it is not clear whether the TSP2 sequence could be recognized by $\alpha_3\beta_1$ integrin. Activity of the latter sequence may be increased in an environment that increases protonation of the imidazole in His.

Previous publications have not identified a consensus $\alpha_3\beta_1$ integrin recognition sequence in its ligands. One hypothesis is that different ligands have unrelated binding sequences, which is supported by a recent mutagenesis study (32). However, other recent data have raised questions about whether all of the proteins reported to mediate $\alpha_3\beta_1$-dependent adhesion are true $\alpha_3\beta_1$ ligands (33). LamA2 and LamA3 were verified to bind $\alpha_3\beta_1$ integrin. These have potential binding motifs based on our data, but human LamA1, which was found not to bind $\alpha_3\beta_1$ with high avidity, has an Ala in the position occupied by the essential Arg in the TSP1 sequence. Substitution of Ala for the Arg in the TSP1 sequence abolished all activity of the synthetic TSP1 peptide. Among the five G domain modules of LamA3, G2 has a better consensus sequence based on our results (NLK) than does G4 (NFQ) or G5 (NIH). Expressed as recombinant proteins, only the G2 module promoted $\alpha_3\beta_1$-dependent adhesion (34). Although RGD was reported to be an $\alpha_3\beta_1$ ligand, the RGD in entactin is not required for recognition, and the RGD in the type 3 repeats of TSP1 is not recognized by this integrin. A binding site for the $\alpha_3\beta_1$ integrin in entactin was mapped to the G2 domain (residues 301–647) (11). Multiple alignment of this region of entactin against the TSP1 sequence and the murine laminin-1 peptide GD6 identified a related sequence, FSGIDEHGHLTI, but this sequence lacks all of the essential residues in the TSP1 sequence. This domain of entactin also contains two NXR sequences: NNRH and NGRQ. It remains to be determined whether either of these can function as an $\alpha_3\beta_1$ integrin recognition sequence.

The absence of an Asp residue in peptide 678 may account for its partial independence of divalent cations. An Asp residue is usually considered an essential element for integrin peptide ligands (35, 36). According to one model for integrin ligand binding, the divalent cation participates directly in binding an Asp-containing peptide ligand (reviewed in Ref. 37). Thus an integrin peptide ligand without a carboxyl side chain cannot coordinate with a bound divalent cation and therefore may not have a divalent cation requirement for binding to the integrin. The alternate model, proposing an indirect role of divalent cations in integrin activation (37), would be consistent with the observed stimulation of cell spreading on peptide 678 by Mn^{2+} but not Ca^{2+} and the partial inhibition following chelation of divalent cations.

Another interpretation of the partial divalent cation independence for the adhesive activity of peptide 678 is that ionic interactions of the Arg side chain in the TSP1 peptide with the negatively charged cell surface contribute to the adhesive activity of this peptide. Weak ionic interactions could promote adhesion to the immobilized peptide through multivalent interactions with negatively charged glycoproteins and proteoglycans on the cell but would not significantly contribute to binding of the same monovalent peptide to the cell in solution. This hypothesis would explain why the Arg-containing peptides 686 and 691, in which the essential Val or Asn residues were substituted with Ala, lacked activity in solution to inhibit adhesion to $\alpha_3\beta_1$ ligands but retained some adhesive activity when immobilized. Thus, inhibitory activities in solution may provide a more reliable assessment of integrin binding specificity for Arg-containing peptides.

Spreading of MDA-MB-435 breast carcinoma cells on intact TSP1 (14) or the $\alpha_3\beta_1$ integrin-binding peptide 678 induces formation of filopodia. In cells plated on peptide 678, these structures are enriched in the α_3 integrin subunit, suggesting that engagement of this integrin by TSP1 triggers formation of filopodia. Formation of filopodia or microspikes has been noted during attachment of other cell types on TSP1 (38). This response may be mediated by the $\alpha_3\beta_1$ integrin, because lamellar spreading rather than formation of filopodia was typically observed on melanoma cells that predominantly use the $\alpha_v\beta_3$ integrin receptor for spreading on TSP1 (26).

Using multiple sequence alignment, the N-terminal domains of thrombospondins were recently shown to contain a module related to pentraxins and to the G domain modules of laminins (39). Based on this alignment, both the $\alpha_3\beta_1$ integrin-binding sequence from TSP1 identified here and the GD6 sequence of laminin are located at the C terminus of a pentraxin module. The known three-dimensional structures of other members of the same superfamily (40, 41) lead to the prediction that both potential integrin binding sequences are located in the last β-strand of a pentraxin module and therefore may be presented with similar topologies on the laminin G domain and the N-terminal domain of TSP1. This observation suggests an evolutionary relationship between the thrombospondin N-terminal domains and laminin G domains that is consistent with their proposed common function as recognition sites for a β_1 integrin receptor.

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Inhibition of Angiogenesis by Thrombospondin-1 Is Mediated by 2 Independent Regions Within the Type 1 Repeats

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Background—Suppression of tumor growth by thrombospondin-1 (TSP-1) has been associated with its ability to inhibit neovascularization. The antiangiogenic activity of TSP-1, as defined by cornea pocket assays, was previously mapped to the amino-terminal portion of the protein within the procollagen region and the type 1 repeats.

Methods and Results—We evaluated the specificity and efficacy of different regions of TSP-1 using recombinant fragments of the protein on chorioallantoic membrane (CAM) angiogenesis and endothelial cell proliferation assays. In both assays, fragments containing the second and third type 1 repeats but not the procollagen region inhibited angiogenesis and endothelial cell proliferation. To further define the sequences responsible for the angiostatic effect of TSP-1, we used synthetic peptides. The CAM assay defined 2 sequences that independently suppressed angiogenesis. The amino-terminal end of the type 1 repeats showed higher potency for inhibiting angiogenesis driven by basic fibroblast growth factor (FGF-2), whereas the second region equally blocked angiogenesis driven by either FGF-2 or vascular endothelial growth factor (VEGF). Modifications of the active peptides revealed the specific amino acids required for the inhibitory response. One sequence included the conserved tryptophan residues in the amino-terminal end of the second and third type 1 repeats, and the other involved the amino acids that follow the CSVTCG sequence in the carboxy-terminus of these repeats. Both inhibition in the CAM assay and inhibition of breast tumor xenograft growth in nude mice were independent of the TGF-β–activating sequence located in the second type 1 repeat.

Conclusions—These results indicate that the type 1 repeats of TSP-1 contain 2 subdomains that may independently inhibit neovascularization. They also identify 2 independent pathways by which TSP-1 can block FGF-2 and VEGF angiogenic signals on endothelial cells. (***Circulation.*** **1999;100:1423-1431.)**

Key Words: angiogenesis ■ endothelium ■ vessels

Thrombospondin (TSP-1) is a matricellular protein with recognized ability to inhibit endothelial cell proliferation and to suppress angiogenesis.[1,2] The region responsible for inhibition of angiogenesis has been mapped to the procollagen domain and to the type 1 repeats.[3] The molecular mechanisms for this inhibition are not entirely understood. It is likely that the inhibition of capillary growth by TSP-1 might be multifactorial and involve competition for basic fibroblast growth factor (FGF-2) binding to the endothelial cell surface,[4,5] binding to heparan sulfate proteoglycans,[4] activation of latent transforming growth factor (TGF)-β_1,[6] and/or binding to CD36, a receptor for TSP-1.[7] More importantly, the ability of TSP-1 to suppress neovascularization has been associated with inhibition of tumor growth.[8–10] We have previously demonstrated in xenograft assays that stable synthetic peptide analogues of the TSP-1 type 1 repeats suppressed breast carcinoma growth in a dose-dependent manner.[10] The peptides used in these assays included a tryptophan-rich motif within the type 1 repeats that binds to heparin.[10,11] However, these peptides did not include the sequence that binds to CD36 and elicited an antiangiogenic effect in the cornea.[3] Additional studies have indicated that these tryptophan-rich peptides suppressed endothelial cell proliferation, inhibited chemotaxis to FGF-2,[4] and induced apoptosis of endothelial cells.[11] Although no direct analyses were performed to elucidate their role in angiogenesis, their suppression of FGF-2–stimulated endothelial proliferation and chemotaxis were indicative of an effect on tumor vascularization.[4] It is not clear how potent these peptides are in relation to other recognized vascular inhibitory regions of TSP-1[7] or whether they might demonstrate variability in efficacy depending on the angiogenic stimulus (ie, FGF-2

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versus vascular endothelial growth factor [VEGF]). The present study was therefore undertaken to (1) evaluate the efficacy of peptides from several regions of the type 1 repeats to inhibit angiogenesis, (2) determine the effect of these peptides on FGF-2– versus VEGF-driven angiogenesis, and (3) elucidate the residues that provide a minimal functional core sequence in TSP-1.

Methods

Protein, Recombinant Fusion Protein, and Peptides

Thrombospondin was purified from human platelets.[12] Recombinant fusion proteins of TSP-1 were prepared by use of the pGEX vectors.[12] Vascular permeability factor (VPF)/VEGF was obtained from Peprotech; FGF-2 was a generous gift from Dr Gera Neufeld (Technion University, Israel).

The peptides used in this study were synthesized on a Biosearch model 9600 peptide synthesizer using standard Merrifield solid-phase synthesis protocols and *t*-butoxycarbonyl chemistry.[10] Peptides were analyzed by reverse-phase high-performance liquid chromatography and further purified by dialysis with Spectrapor 500 MWCO tubing, gel permeation chromatography, or reverse-phase purification with C18 Sep-pak cartridges. The identities of peptides were verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Polysucrose conjugates of some peptides were prepared as previously described.[10] Before use on chorioallantoic membrane (CAM) assays, peptides were also filtered on Centricon P100 to eliminate traces of endotoxin.

Endothelial Cells and Proliferation Assays

Chicken endothelial cells (CECs) were isolated from the brain of day 7 chicken embryos. Endothelial cells were purified and characterized by standard techniques, as described.[13]

For proliferation assays, quiescent endothelial cells were seeded in 24-well plates in EBM medium supplemented with 0.1% FCS and 50 ng/mL of VEGF (PeproTech Inc) and/or 2 ng/mL of FGF-2 in the presence of TSP-1, fusion proteins, peptides, or vehicle control. During the last 8 hours of the treatment, cells were pulsed with 1 μCi/well of [^{3}H]thymidine (DuPont-NEN). Trichloroacetic acid–precipitable counts of [^{3}H]thymidine were measured as previously described.[14] The significance of inhibition was assessed by a 2-tailed *t* test.

CAM Assays

The effect of TSP-1, fusion proteins, and peptides on angiogenesis was evaluated with a modified CAM assay.[14] The method is based on the vertical growth of new capillary vessels into a collagen gel pellet placed on the CAM. The collagen gel was supplemented with an angiogenic factor such as FGF-2 (50 ng/gel) or VEGF (250 ng/gel) in the presence or absence of test proteins/peptides. The extent of the angiogenic response was measured by use of FITC-dextran (50 μg/mL) (Sigma) injected into the circulation of the CAM. The degree of fluorescence intensity parallels variations in capillary density; the linearity of this correlation can be observed with a range of capillaries between 5 and 540. Morphometric analyses were done by acquisition of images with a Sony, single-chip CCD camera. Images were imported into NHImage 1.59, and measurements of fluorescence intensity were obtained as positive pixels. Each data point was compared with its own positive and negative controls present in the same CAM and interpreted as percentage of inhibition, considering the positive control to be 100% (VEGF or FGF-2 alone) and the negative control (vehicle alone) 0%. Statistical evaluation of the data was performed to check whether groups differ significantly from random by analysis of contingency with Yates' correction.

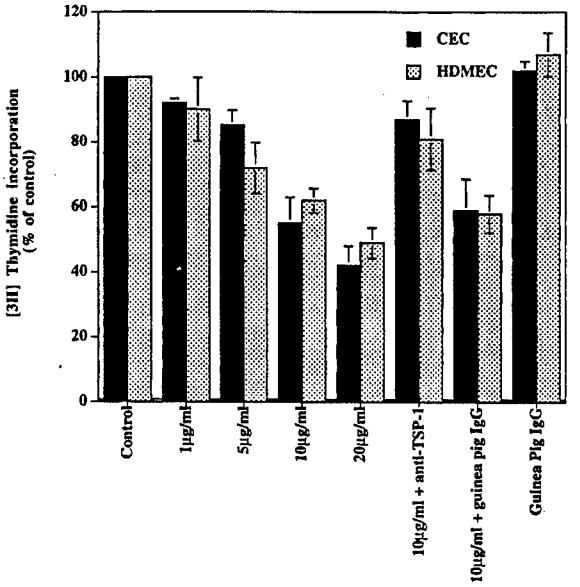


Figure 1. Inhibition of endothelial proliferation by human TSP-1. Quiescent CECs and human dermal microvascular endothelial cells (HDMEC) were seeded on 24-well plates and stimulated to proliferate by addition of 2 ng/mL FGF-2 and 10 ng/mL of VEGF and in presence of TSP-1 (at indicated doses), TSP-1 antibodies, or control IgG alone for 48 hours. A pulse of [^{3}H]thymidine (1 μCi/mL) was given for last 8 hours of treatment. Experiments were performed 4 times with independent preparations of TSP-1 and in triplicate. For direct comparisons between 2 cell types, values were converted to percentage of control. Values at ≥5 μg/mL were statistically different from control in both cell types.

Tumorigenesis Assay in Nude Mice

NIH nu/nu mice ≈8 weeks old were injected with 105 MDA-MB-435 cells by the mammary fat pad route as previously described.[10] Eight animals were injected for each condition. Beginning at day 25 and continuing every day until day 40, the animals were injected intravenously (tail vein) with 100 μL of the free peptide (6 mg/kg) in HBSS or with HBSS alone. The animals were euthanized at day 60. The primary tumors were removed, stripped free of other tissues, weighed, and fixed in formaldehyde.

Results

To validate the use of the chicken CAM for assessing angiogenic responses to human TSP-1, we first determined whether human TSP-1 inhibited the proliferation of CECs. Figure 1 shows that human TSP-1 inhibited the proliferation of CECs to a degree similar to that of human dermal microvascular endothelial cells.

The possible contamination of platelet TSP-1 preparations with TGF-β_1 raises concerns about the contribution of TGF-β to the antiproliferative activities of platelet TSP-1.[6] TGF-β has been shown to bind and to copurify with TSP-1.[6] Furthermore, TGF-β is a potent inhibitor of endothelial cell proliferation.[15] The specificity of TSP-1–mediated suppression of endothelial cell proliferation was validated with anti–TSP-1 blocking antibodies (Figure 1). A polyclonal TSP-1 antibody raised in guinea pig neutralized the TSP-1–mediated inhibitory effect, whereas antibodies alone had a mild but statistically insignificant effect on proliferation (data not shown). Preimmune guinea pig IgG in the presence of TSP-1 did not ameliorate endothelial growth inhibition. We also examined the levels of TGF-β_1 present in the TSP-1

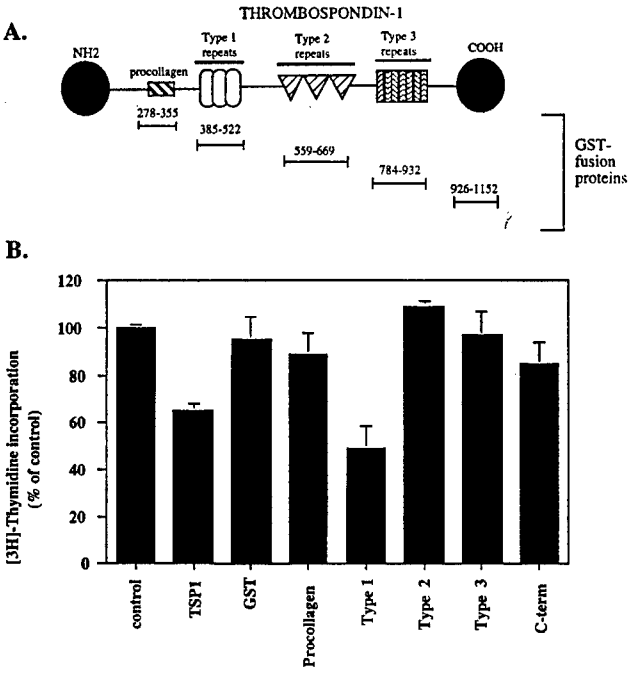


Figure 2. Inhibition of CEC proliferation by recombinant fragments of TSP-1. A, Schematic of 180-kDa TSP-1 monomer and distribution of GST–TSP-1 fusion proteins. Amino acid residues included in each fusion protein are numbered from the amino-terminal asparagine residue and are procollagen, 278 to 355; type 1 repeat, 385 to 522; type 2 repeat, 559 to 669; type 3 repeat, 784 to 932; and carboxy-terminal, 926 to 1152. B, TSP-1 (at 25 μg/mL) and GST–TSP-1 fusion proteins (at 10 μmol/L) were evaluated on proliferation assays. Values are mean±SD of triplicate wells and are presented as percent of control (growth factors and vehicle). Only TSP-1 and type 1 repeat fusion proteins are significantly different from control ($P<0.005$).

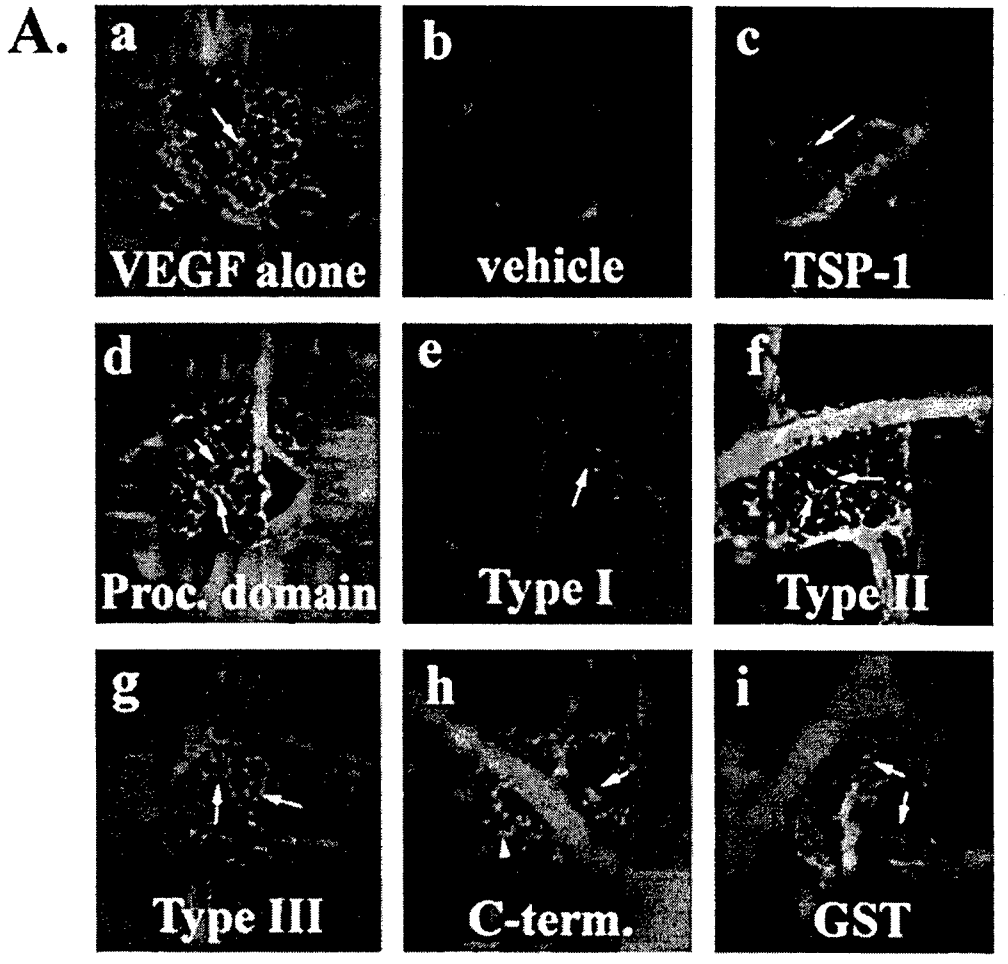
preparations. In general, TGF-β ranged between 5 and 90 pg/mL in our preparations of TSP-1 from platelets. Further dilution in tissue culture media reduced this value 100- to 500-fold in the proliferation assays. Addition of purified TGF-β to the CECs suppressed proliferation only at 5 ng/mL (data not shown). Taken together, the neutralization of the antiproliferative effect mediated by anti–TSP-1 antibodies and the limited levels of TGF-β contamination confirm that the suppressive effects on endothelial proliferation were mediated by TSP-1.

The specificity of TSP-1 to inhibit CEC proliferation was also determined by use of glutathione *S*-transferase (GST) fusion proteins expressing the procollagen, type 1, type 2, type 3, and C-terminal domains of TSP (Figure 2). These bacterially expressed proteins lack any TGF-β contamination and can be used to define the functional domain(s) within TSP-1 responsible for the inhibitory effect. Interestingly, only the type 1 repeat recombinant fragment inhibited endothelial cell proliferation. TSP-1 10 μmol/L inhibited proliferation by 35% on CECs ($P=0.009$). At the same molar concentration, the recombinant GST type 1 domain inhibited CEC proliferation by 48% ($P=0.0009$). The GST control alone had no effect. Other recombinant fragments tested in this assay did not show inhibition of either bovine aortic endothelial cell (data not shown) or CEC proliferation, and the partial inhibition by the carboxy-terminal fusion protein (83% of control) was not statistically significant ($P=0.21$). It was interesting that the entire TSP-1 molecule had a more moderate effect on proliferation than that of the GST fusion protein containing the type 1 repeats.

The effects of intact TSP-1 and GST fusion proteins on angiogenesis were evaluated on a mesh CAM assay. Growth of capillaries in this assay is stimulated vertically, against gravity, by VEGF or FGF-2 cast into a polymerized collagen gel. Figure 3A shows the effect of TSP-1 and fusion proteins on neovascularization of the acellular collagen matrix. The presence of angiogenic growth factors induces the growth of a thin vasculature in the acellular gel as early as 24 hours (Figure 3A, arrows) from the thicker vessels located under the nylon mesh (larger vessels out of focus). In the absence of angiogenic growth factors, no network was observed (Figure 3B). The ability of inhibitors to suppress the stimulatory signal of growth factors was then evaluated by inclusion of these proteins in the polymerized vitrogen gel. Both TSP-1 and the GST type 1 repeat fusion protein were effective at suppressing the angiogenic response mediated by growth factors. TSP-1 was able to block VEGF-mediated angiogenesis by 35%, whereas the GST type 1 repeat fusion protein was more effective at the same molar ratio (57%) (Figure 3B). No significant effects were detected with any of the proteins alone, ie, in the absence of VEGF, or with proteins other than the type 1 repeats in the presence of VEGF or FGF-2. The effect was reproducible with several preparations of TSP-1 and of recombinant protein and was performed at least 4 separate times with each treatment in triplicate (total of 12 assays). These results are consistent with data obtained from the proliferation experiments and again indicate that at an equivalent molar ratio, the type 1 repeats of TSP-1 appear to be more effective than the intact protein. To this end, it has recently been postulated that the carboxy-terminal end of TSP-1 might exert a positive effect on angiogenesis by its ability to interact with integrin-associated protein.[16] Interestingly, we observed a slight but reproducible increase in angiogenic rate with the carboxy-terminal end (Figure 3B). The entire TSP-1 protein might therefore contain regions that elicit both positive and negative signals on endothelial cell proliferation and angiogenesis, thus providing amelioration to the suppressive growth signals. A careful dissection of these areas is required to clearly elucidate the potential function of each domain.

We also found that the first repeat of the type 1 domain had no effect on suppressing angiogenesis in the CAM assay (Figure 3B). The ability of TSP-1 to inhibit vascular growth therefore appears to be located within the last 2 type 1 repeats.

We next examined the activities of synthetic peptides derived from the procollagen and second and third type 1 repeats of TSP-1, as well as the carboxy-terminal end of TSP-1 (Figure 4 and Table). The procollagen region and the last 2 type 1 repeats have previously been shown to have antiangiogenic activity in the cornea pocket assay.[3] Evaluation of the procollagen region in the CAM assay, however, did not result in angiogenic suppression, in contrast to peptides from the second (508) and third (616) type 1 repeats,



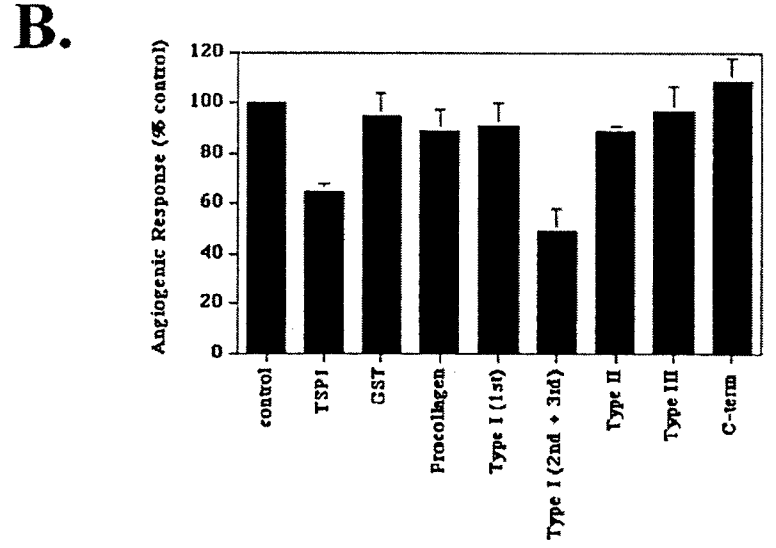


Figure 3. Effect of TSP-1 fusion proteins on angiogenesis. A, Mesh-CAM assays for evaluation of TSP-1 and GST fusion proteins were performed on day 11 chicken embryos. Each pellet contained vitrogen 50 μg/mL, VEGF 250 ng/mesh, and FGF-2 50 ng/mL (a), except negative control (no growth factors were added) (b), in addition to TSP-1 (c), GST-procollagen region (d), GST type 1 repeats (e), GST type-2 repeats (f), GST type-3 repeats (g), GST carboxy-terminus (h), or GST control (i). Fusion proteins were used at 20 μmol/L and TSP-1 at 10 μg. B, Quantification of angiogenic response. Evaluation was determined 24 hours after application of pellets to CAM surface. Systemic injection of FITC-dextran revealed vessels with patent lumens. Ten squares of 250 μm^2 were evaluated per mesh. Three meshes in independent embryos were performed per time point. In each case, extent of angiogenesis suppression/stimulation was determined by direct comparison to control meshes (VEGF alone=100%) in same CAM. As in Figure 2, only TSP-1 and type 1 repeat fusion proteins were significantly different from control ($P<0.01$).

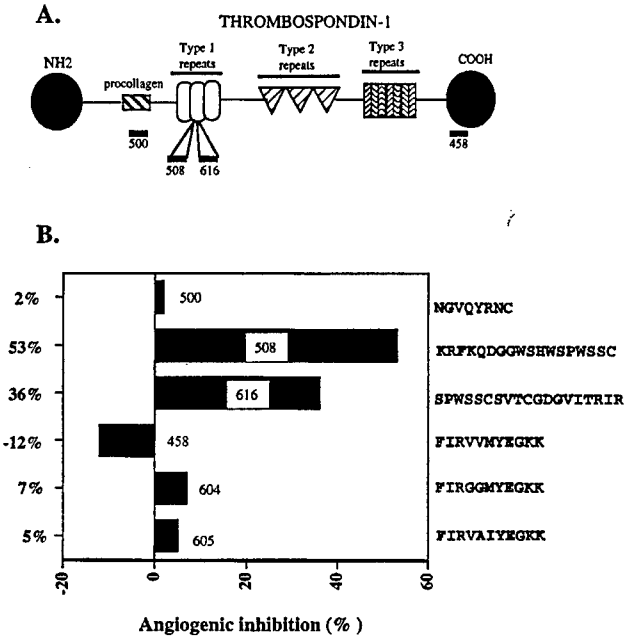


Figure 4. Evaluation of TSP-1 peptides from procollagen, type I repeats, and carboxy-terminal regions on CAM assays. A, Schematic of TSP-1 and location of peptides used in B. B, Assessment of angiogenic response was determined as in Figure 3. Wild-type peptides from procollagen region (500F), type I repeats (508, 616), and carboxy-terminus (458) were evaluated at 10 μmol/L. In addition, mutated peptides from carboxy-terminus were tested as well (604 and 605). Note that negative values are read as angiogenic response above control, ie, growth factors alone.

Peptides Used in the Assays

No.	Peptide Sequence
500F	NGVQYRNC-polysucrose
508	rv-amKRFKQDGGWSHWSPWSSC-ac
545	rv-amKRAKQAGGWSHWSPWSS-ac
416F	rv-amKRFKQDGGWSHWSPWSSC-ac-polysucrose
500F	NGVQYRNC-polysucrose
493	rv-amGGWSHWSPWAAtp
530	rv-amKRFKQAGWSHWAA-ac
553	rv-amKRAKQAGWSHWAA-ac
571	rv-amAAWSHWA-ac
596	rv-amKRAKQAGGWSHWSPWSS-ac
597	rv-amKRFKQDGGASHASPASS-ac
598	rv-amKRAKQAGGASHASPASS-ac
599	rv-amKRFKQAGGWSHWSPWSS-ac
187	CSVTCG
203	VTCG
205	VTCGDGVITR
237	SSVTCG
245	VTCGGGVQKRSRL
246	KRFKQDGGWSHWSPWSS
535	TRIRQDGGWSHW
600	SSWPSWHSWGGDQKFRK
616	SPWSSCSVTCGDGVITRIR
458	FIRVVMYEGKK
604	FIRGGMYEGKK
605	FIRVAIYEGKK

which were antiangiogenic. Interestingly, a peptide from the carboxy-terminal domain (458) showed a slight but reproducible proangiogenic effect. The region has been shown to interact with integrin-associated protein and enhance attachment and migration.[16] The specificity of this positive response was supported by concurrent experiments performed with 2 mutated versions (604 and 605) of the carboxy-terminal peptide. Substitution of the 2 Val residues was sufficient to suppress the proangiogenic effect (Figure 4).

On the basis of these studies, it appears that in the CAM assay, only peptides derived from the second and third type 1 repeats are angioinhibitory. We therefore focused subsequent studies on these domains.

Figure 5 shows the sequence of the last 2 TSP-1 type 1 repeats and correlates previously identified functions to specific sequences within these domains. A region of interest, because of its demonstrated ability to suppress tumor growth, is located at the amino-terminal end of these repeats.[10] The direct effect of this region on angiogenesis has not yet been tested. Our results indicated that peptides from this region at 1 μmol/L suppress vascular growth induced by a mixture of FGF-2 and VEGF (Figure 6A). The effect was dose-dependent, and retro-inverso analogues or polysucrose conjugates of the peptides were more potent than the native TSP-1 peptide. These modifications are known to increase the half-life of polypeptides by reducing degradation in vivo.[17]

Assays were also performed using mutated versions of the peptides as well as deletion mutants. Mutation of the 3

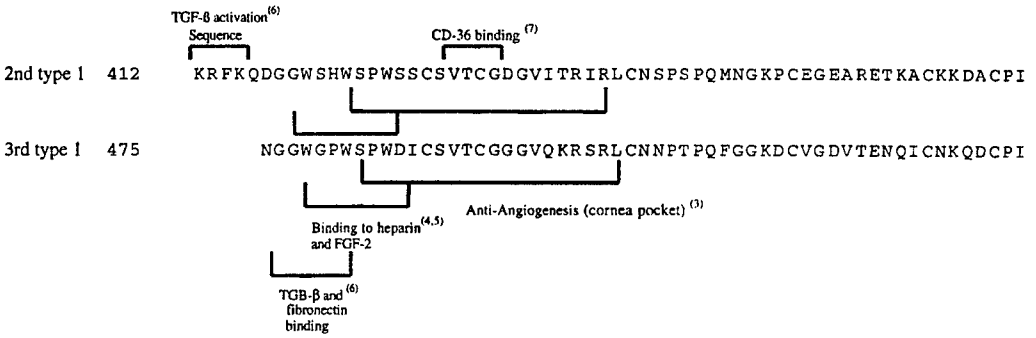


Figure 5. Amino acid sequence of second and third type 1 repeats of TSP-1. Amino acids are given in single-letter code. Regions with identified activity are indicated. References are provided (superscript numbers).

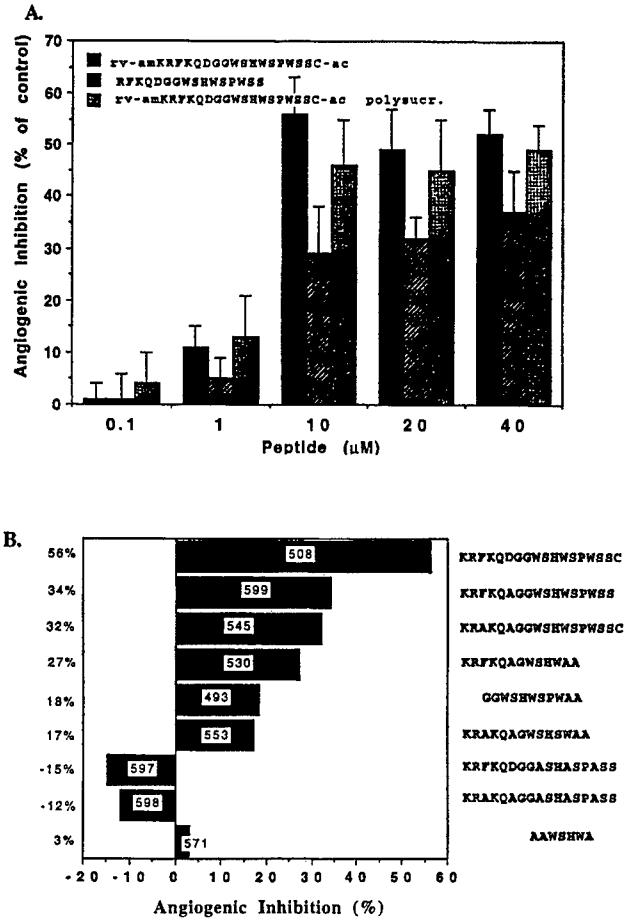


Figure 6. Effect of amino-terminal region of type 1 repeats on angiogenesis. Evaluation of angiogenic response was determined as in Figure 3. Peptides used were wild-type and mutated versions of subdomains located within second and third type 1 repeats; refer to Figure 5 for orientation. A, Dose responses for inhibition of angiogenesis by natural L-forward and D-reverse TSP-1 peptides from second type 1 repeat and a polysucrose conjugate of D-reverse sequence. Statistical analysis (*t* test) revealed no significant differences among wild-type peptides. Error bars=±SD. B, Evaluation of deletions and mutated versions of wild-type peptides (10 μmol/L). All sequences were tested as D-reverse peptides. Corresponding forward sequences are indicated on right. Note that mutation of tryptophan residues completely abolished antiangiogenic activity of peptides.

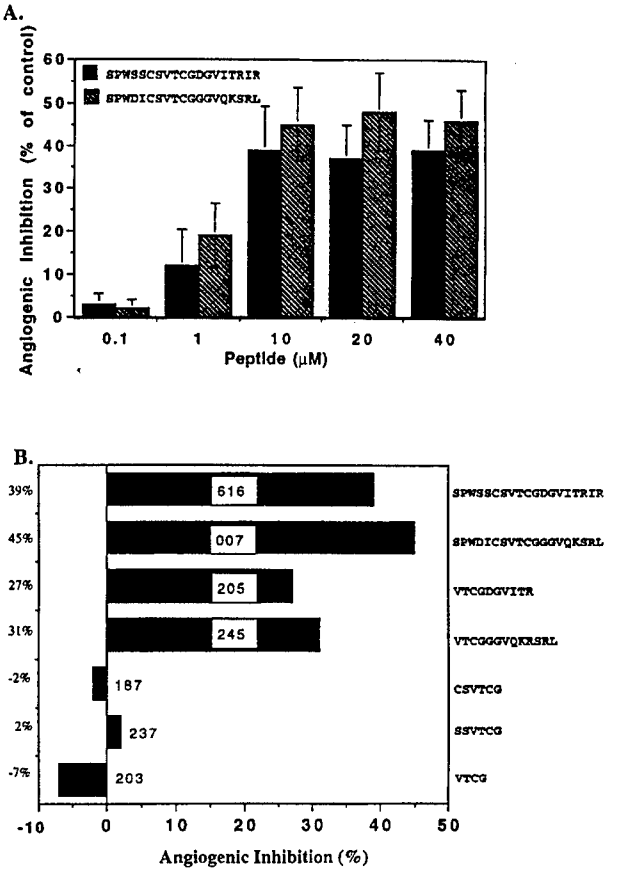


Figure 7. Effect of carboxy-terminal half/CD36-binding region of type 1 repeats on angiogenesis. Angiogenic response to peptides (refer to Figure 5 for orientation) was determined as in Figure 3. A, Dose curve of L-forward peptides from second and third type 1 repeats. Statistical analysis (*t* test) revealed no significant differences between peptides derived from second and third type 1 repeats. Error bars=±SD. B, Evaluation of deletions and mutated versions of wild-type peptides (10 nmol/L). Sequences are indicated on right. Major activity for both peptides is located carboxy-terminal from VTCG sequence, a region recognized for its interaction with CD36.

tryptophan residues to alanines (peptides 597 and 598) completely abolished inhibitory activity, indicating that these residues are critical to the antiangiogenic response (Figure 6B). A peptide with 2 instead of 3 tryptophan residues was partially effective (peptides 493 and 530) (Figure 6B). The latent TGF-β–activating sequence (RFK) was not required for the suppressive effect on neovascularization, because mutation of the essential phenylalanine residue to alanine (compare peptides 545 and 596) did not have any deleterious effect on the activity of the peptide. This mutation abrogates the ability of this peptide to activate latent TGF-β_1.[6]

We next examined the effects of the second half of the type 1 repeats, a region previously concluded to be responsible for the antiangiogenic effects promoted by TSP-1 in cornea pocket assays.[3] Our results support those observations and demonstrate activity of the same peptides in the CAM assay (Figure 7A). In agreement with recent reports,[7] the active region appears to be carboxy-terminal to the CSVTCG region, because this sequence alone was inactive, and deletion of the first 2 residues of this motif did not affect the antiangiogenic activity of peptide 205 (VTCGDGVITR) from the second type 1 repeat or peptide 245 (VTCGGGVQKRSRL) from the third type 1 repeat (Figure 7A). However, the VTCG sequence without these flanking sequences completely lacked antiangiogenic activity. This flanking sequence has been shown to act through CD36, a receptor for TSP-1.[7] We have verified that CECs and vessels in the CAM do express this receptor by Northern blot analysis (data not shown). Therefore, it is likely that the mechanism of action is similar.

To further elucidate the mechanism of action of these 2 subregions, CAM experiments were performed with either VEGF or FGF-2 as stimulator of the angiogenic response (Figure 8). Interestingly, we observed a clear distinction between the tryptophan repeat peptides and the CD36-binding domain peptides. Peptides 508 and 599, which have

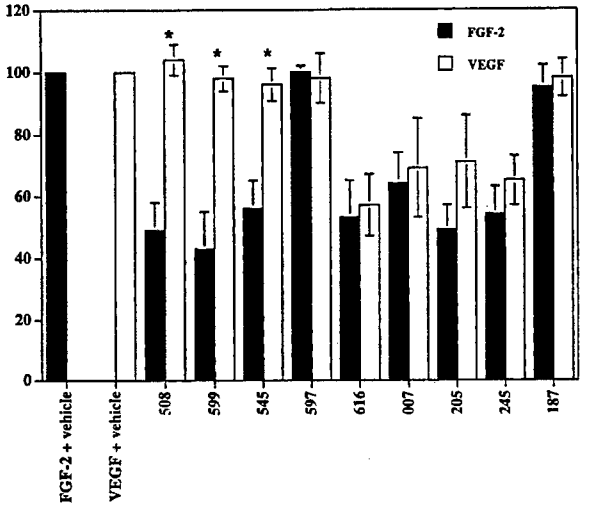


Figure 8. Effect of TSP-1 peptides on FGF-2 and VEGF-induced angiogenesis. Angiogenesis in gel pellet (50 μL) was induced with FGF-2 (50 ng) supplemented in gels (solid bars) or VEGF (250 ng) (open bars). Quantification was normalized to level of angiogenic response driven by each growth factor alone (=100%). Error bars represent SD of quadruplicate experiments performed in independent CAMs. *$P<0.01$.

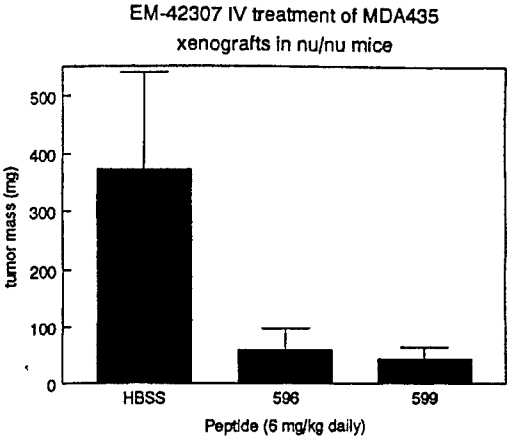


Figure 9. Inhibition of MDA MB435 breast carcinoma tumor growth by TSP-1 peptide analogues. Groups of 8 nu/nu mice received implants in mammary fat pad 4 of 1×10^5 MDA435 cells on day 0. From day 25 through day 40, animals were injected daily with 0.1 mL HBSS or HBSS containing retro-inverso TSP-1 peptide analogues (599 or 596) at a dose of 6 mg $\cdot$ kg^{-1} $\cdot$ d^{-1}. Tumor mass is presented as mean$\pm$SD for each experimental group.

the tryptophan motif, suppressed the angiogenic response only to FGF-2–mediated angiogenesis but had no effect on VEGF-driven vascular growth. In contrast, the CD36-binding sequences blocked both VEGF- and FGF-2–induced angiogenesis. Interestingly, the tryptophan domain has previously been shown to prevent FGF-2 binding to endothelial cells.[4,5] Therefore, inhibition of binding or further sequestration of FGF-2 is the most likely mechanism of action of this amino-terminal portion of the type 1 repeats.

A surprising result was the lack of antiangiogenic activity of the TGF-β activating sequence. To determine whether the CAM assay has predictive value for inhibition of tumor angiogenesis, we assessed the role of the TGF-β_1–activating sequence in inhibition of breast carcinoma tumor growth in vivo using orthotopic xenografts of MDA435 breast carcinoma cells in athymic mice (Figure 9). D-Reverse analogues of the native TSP-1 sequence (peptide 599) and a modified sequence lacking TGF-β–activating activity (peptide 596)[10] both strongly suppressed tumor growth when administered intravenously to the mice beginning 25 days after implantation of the tumor cells in the mammary fat pad. The data are in agreement with our findings in the CAM assay and provide further support that the suppression of tumor growth results from the ability of TSP-1 to suppress angiogenesis independently of latent TGF-β activation.

Discussion

In this study, we evaluated both TSP-1 fusion proteins and synthetic peptides to provide a comparative assessment of the antiangiogenic activity displayed by the different domains of TSP-1. We combined these data with analysis of deletion and point mutants to identify the minimal sequences with vasculoinhibitory properties. One result of these structure/function analyses demonstrated that the ability of TSP-1 to suppress angiogenesis resides solely in the second and third type 1 repeats. The data are in agreement with previous findings by Tolsma and coworkers.[3] In addition, these studies revealed 2 subdomains in the type 1 repeats that act independently to suppress angiogenesis and identified essential residues responsible for these effects: the tryptophan-rich WSXW motifs and the CD36-binding region. These 2 sequences also display growth factor selectivity in that the tryptophan-rich domain preferentially suppresses FGF-2 angiogenic signals, whereas the CD36-binding region inhibits capillary formation driven by either FGF-2 or VEGF. These findings contribute to understanding the antitumor activity of some TSP-1 peptides and suggest that these peptides might act, at least in part, by suppressing tumor-mediated angiogenesis.

The neovascular suppression displayed by TSP-1 has previously been attributed to the second (amino acid [aa] 424 to 442) and third (aa 481 to 246) type 1 repeats of TSP-1.[3] The type 1 domain of TSP-1 consists of 3 polypeptide repeats that have complete conservation of the cysteine and tryptophan residues and that have been identified in a variety of other proteins, including properdin, F-spondin, BAI, and metallospondins.[18–21] Nevertheless, the antiangiogenic potential of the type 1 repeats is not shared by all these proteins, indicating that context-specific primary sequences and/or secondary structure influence the function of the type 1 repeats.

Previous publications have indicated that the first type 1 repeat of TSP-1 has no antiangiogenic activity.[3] We confirmed these results in the CAM angiogenic assay. Recent studies have further mapped a subregion within the second and third type 1 repeats including and carboxy-terminal to the CSVTG sequence with angioinhibitory effects.[7] The relevant region has been found to bind to CD36 and to be responsible for the intracellular events related to the suppression of several mitogenic signals on endothelial cells.[7] Our data are consistent with the observations of Dawson et al[7] but do not correlate with evidence that the CSVTCG sequence alone,

responsible for binding of TSP-1 to CD36, has an effect.[22] Further structure-function and mutagenesis analysis will be required to resolve these discrepancies.

Although activity of the CD36-binding peptides from the carboxyl end of the second and third type 1 repeats provides an explanation for some of the activity of the type 1 repeats, other regions within the type 1 repeats have also been shown to reduce tumor growth and display potential angiostatic or antiangiogenic activities.[10,11] Because these small fragments of the protein were tested in different laboratories and angiogenesis assays, we felt that it was necessary to analyze TSP-1 fragments and peptides side by side and in a single in vivo assay. Our observations demonstrate that the tryptophan-rich motif contains a second angioinhibitory region with activity similar to that of the CD36-binding sequence. We would predict that the potential of this region to inhibit FGF-2–mediated angiogenesis relates to its ability to bind to heparan sulfate and thereby block FGF-2 receptor signaling.[5] The minimum sequence necessary for heparin-binding activity is the pentapeptide WSPWS, although if the preceding positively charged residues are added to the SHWSPWSS sequence, the heparin-binding activity of the peptide can be enhanced up to 10-fold.[10] Our results indicate that the GGWSHWSPWSS worked better for inhibiting angiogenesis than the SHWSPW sequence alone.

The interaction of the type 1 repeats with heparin in the tryptophan-rich region lacks stereospecificity, because forward and inverse peptide analogues (L-forward, L-reverse, and D-reverse) displayed equivalent ability to interact with heparin, and in the CAM angiogenesis assay, the retro-inverso analogue was better in blocking angiogenesis. Thus, the polypeptide backbone is not involved in this response. Conjugation of the type 1 repeat peptides to polysucrose did not significantly affect their antiangiogenic function, although conjugation increased their potency in vitro for inhibiting proliferation of endothelial and breast carcinoma cells stimulated by FGF-2.[11] The D-reverse analogues are resistant to proteases, and we have shown in xenograft assays that retro-inverso analogues are effective when administered intravenously in mice.[10] The enhanced activity of the D-reverse peptides in the CAM assay may therefore result from an enhanced half-life in the gel or chick embryo circulation.

Studies by Tolsma and coworkers[3] have demonstrated that in addition to the type 1 repeats, a region in the procollagen domain (aa 294 to 317) inhibits angiogenesis in the cornea pocket assay. Our results, however, did not support these findings by use of either the fusion protein or the synthetic peptide. It is possible that unlike with the type 1 repeats, the procollagen region is not effective across species; in fact, the amino-terminal end of TSP-1 differs more significantly than the carboxy-terminal end.[2] Nevertheless, we also were not able to see any effect on proliferation or migration using the procollagen region fusion protein on human dermal microvascular endothelial cells.

TSP-1 has been shown to bind and activate latent TGF-β_1.[6] Because TGF-β modulates endothelial cell function,[15,23,24] some involvement of the latent TGF-β–activating sequence was expected in the activity of TSP-1. Although TGF-β inhibits endothelial cell proliferation,[15] angiogenesis assays in vitro[23] as well as injection of TGF-β in vivo have demonstrated proangiogenic activity.[24] Peptides with KRFK sequences might therefore be predicted to induce angiogenesis by activating endogenous latent TGF-β. Perhaps this is why some of the KRFK peptides without the tryptophans seem to stimulate angiogenesis (eg, 597). In any case, our studies demonstrate that at least the antiangiogenic activity of type 1 repeats was independent of latent TGF-β_1 activation, because substitution of an Ala residue for the essential Phe residue did not affect the antiangiogenic potential of the peptide..

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Appendix G (pp 90-131)

Modulation of Endothelial Cell Behavior and Angiogenesis by an α3β1 Integrin-binding Sequence in Thrombospondin-1

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Condensed title: α3β1 Integrin is an Endothelial Thrombospondin Receptor

Keywords: angiogenesis, pathologic; thrombospondins; integrin beta 1; cell motility; peptides

Abstract

Several peptide sequences in thrombospondin-1 (TSP1) have been reported to regulate endothelial cell behavior and inhibit angiogenesis by interacting with endothelial cell CD36 or heparan sulfate proteoglycan receptors. We have now identified a TSP1 peptide that modulates angiogenesis and the *in vitro* behavior of endothelial cells through binding to α3β1 integrin. Recognition of this sequence and intact TSP1 by normal endothelial cells is induced following loss of cell-cell contact or ligation of CD98. Although confluent endothelial cells do not spread on a TSP1 substrate, efficient spreading on TSP1 substrates of endothelial cells maintained without cell-cell contact is mediated by the α3β1 integrin. In solution, an α3β1 integrin-binding peptide from TSP1 inhibits proliferation of sparse endothelial cell cultures, but the same peptide immobilized on the substratum promotes their proliferation. Intact TSP1 also selectively promotes endothelial cell proliferation when immobilized on the substratum. The TSP1 peptide, when added in solution, specifically inhibits migration of endothelial cells into scratch wounds and inhibits angiogenesis in the chick chorioallantoic membrane assay. Thus, recognition of immobilized TSP1 by α3β1 integrin may increase endothelial cell proliferation and motility during wound repair and angiogenesis. Peptides that inhibit this interaction are a novel class of angiogenesis inhibitors.

Angiogenesis under normal and pathological conditions is regulated by both positive and negative signals received from soluble growth factors and components of the extracellular matrix (reviewed in (Folkman, 1995; Hanahan and Folkman, 1996; Polverini, 1995)).

Thrombospondins are a family of extracellular matrix proteins that have diverse effects on cell adhesion, motility, proliferation and survival (reviewed in (Bornstein, 1992; Bornstein, 1995; Roberts, 1996)). Two members of this family, TSP1 and thrombospondin-2, are inhibitors of angiogenesis (Good et al., 1990; Volpert et al., 1995). TSP1 inhibits growth, sprouting, and motility responses of endothelial cells *in vitro* (Canfield and Schor, 1995; Good et al., 1990; Iruela Arispe et al., 1991; Taraboletti et al., 1990; Tolsma et al., 1997) and, under defined conditions, induces programmed cell death in endothelial cells (Guo et al., 1997b). TSP1 inhibits angiogenesis *in vivo* in the rat corneal pocket and chick chorioallantoic membrane angiogenesis assays (Good et al., 1990; Iruela-Arispe et al., 1999). The ability of TSP1 over-expression to suppress tumor growth and neovascularization in several tumor xenograft models provides further evidence for an anti-angiogenic activity of TSP1 (Dameron et al., 1994; Hsu et al., 1996; Sheibani and Frazier, 1995; Weinstat-Saslow et al., 1994). Circulating TSP1 may also inhibit neovascularization of micrometastases in some cancers (Morelli et al., 1998; Volpert et al., 1998). A few studies, however, have concluded that TSP1 also has pro-angiogenic activities under specific conditions (BenEzra et al., 1993; Nicosia and Tuszynski, 1994). Observations of elevated TSP1 expression during endothelial injury and wound repair are also difficult to rationalize with a purely anti-angiogenic activity for TSP1 (Munjal et al., 1990; Reed et al., 1995; Vischer et al., 1988). These apparently contradictory reports have led to confusion about the physiological role of TSP1 as an angiogenesis regulator.

To understand the factors that control the complex responses of endothelium to TSP1, we must define the receptors and signaling pathways that mediate its actions. TSP1 interacts with several receptors on endothelial cells, including the αvβ3 integrin (Lawler et al., 1988), heparan sulfate proteoglycans (Vischer et al., 1997), CD36 (Dawson et al., 1997), the low density lipoprotein receptor-related protein (Godyna et al., 1995), and CD47 (Gao et al., 1996). TSP1 peptides that bind to CD36 or to heparan sulfate proteoglycans inhibit endothelial responses to growth factors *in vitro* and angiogenesis *in vivo* (Iruela-Arispe et al., 1999; Tolsma et al., 1993; Vogel et al., 1993). CD36 expression is required for TSP1 to inhibit the motility response of bovine and human endothelial cells stimulated by FGF2 (Dawson et al., 1997). However, proliferation of several cell types that do not express CD36, including large vessel endothelial cells, are also inhibited by TSP1 and heparin-binding peptides from TSP1 (Guo et al., 1997a; Guo et al., 1998). Based on activities in the chorioallantoic membrane angiogenesis assay, both of these TSP1 sequences can inhibit angiogenesis *in vivo* (Iruela-Arispe et al., 1999). Finally, a sequence from the N-terminal domain of TSP1 can disrupt focal adhesions in endothelial cells, but the effects of this response on angiogenesis have not been defined (Murphy-Ullrich et al., 1993).

TSP1 may also influence angiogenesis indirectly through activation of latent TGFβ (Schultz-Cherry and Murphy-Ullrich, 1993), which in turn can either stimulate or inhibit angiogenesis (Passaniti et al., 1992; Roberts et al., 1986). Based on differences in the phenotypes of *thbs1* and *tgfβ1* null mice and the inability of TGFβ antagonists to block many activities of TSP1 *in vitro*, activation of latent TGFβ probably mediates only a subset of endothelial responses to TSP1 (Crawford et al., 1998).

Integrin interactions are also known to regulate angiogenesis (Brooks et al., 1994). Antagonists of the αvβ3 integrin are potent inhibitors to neovascularization induced by growth factors or in tumors (Brooks et al., 1995). Although αvβ3 is a known TSP1 receptor on endothelial cells (Lawler et al., 1988), its role in modulation of angiogenesis by TSP1 has not been defined. The CD47-binding sequence in TSP1 may increase binding of αvβ3 integrin ligands, including TSP1 itself (Gao et al., 1996; Sipes et al., 1999). However, a recombinant fragment of TSP1 containing the type 3 repeats that bind to αvβ3 did not inhibit angiogenesis (Iruela-Arispe et al., 1999), suggesting that the RGD sequence in TSP1 is not involved in its effects on angiogenesis.

TSP1 interacts with several β1 integrins, including α4β1 and α5β1 on T lymphocytes (Yabkowitz et al., 1993), α3β1 on neurons (DeFreitas et al., 1995), and α3β1 and α4β1 on breast carcinoma cells (Chandrasekaran et al., 1999; Krutzsch et al., 1999). The α3β1 integrin is localized in cell-cell junctions of endothelial cells in a complex with some tetraspan family proteins (Yanez-Mo et al., 1998). Antibodies to several components of this complex, including the α3β1 integrin, inhibited endothelial cell motility in wound repair assays (Yanez-Mo et al., 1998). Based on this observation and our recent finding that recognition of TSP1 by the α3β1 integrin is tightly regulated in breast carcinoma cells (Chandrasekaran et al., 1999), we have examined the role of this integrin in the responses of endothelial cells to TSP1 and regulation of angiogenesis. We demonstrate here that recognition of TSP1 by endothelial cell α3β1 integrin is selectively induced following loss of cell-cell contact. These cells efficiently spread on immobilized TSP1, and this interaction stimulates endothelial cell proliferation. An α3β1 integrin-binding peptide from the amino-terminal domain of TSP1 (Krutzsch et al., 1999) also

modulates endothelial cell proliferation and is a potent inhibitor of endothelial wound repair *in vitro* and angiogenesis *in vivo*.

Materials and Methods

Proteins and Peptides

TSP1 and plasma fibronectin were purified from human platelets or plasma obtained from the NIH Blood Bank (Akiyama and Yamada, 1985; Roberts et al., 1994). Human vitronectin was obtained from Sigma, and bovine type I collagen was obtained from Becton Dickinson Labware Division. Synthetic peptides from TSP1 that are recognized by the α3β1 integrin and structural analogs defective in α3β1 integrin binding were prepared as previously described (Guo et al., 1992; Krutzsch et al., 1999), and GRGDSP was obtained from Gibco/BRL. A non-peptide antagonist of αvβ3 integrin was provided by Dr. William H. Miller (SmithKline Beecham Pharmaceuticals, King of Prussia, PA) (Keenan et al., 1997).

Cells and culture

Bovine aortic endothelial (BAE) cells were isolated from fresh bovine aortae and were used at passages 3-10. BAE cells were maintained at 37° in 5% CO_2 in DMEM (low glucose) medium, containing 10% FCS, 4 mM glutamine, 25 μg/ml ascorbic acid, and 500 U/ml each of penicillin G potassium and streptomycin sulfate. Media components were obtained from Biofluids Inc., Rockville, MD. Primary human umbilical vein endothelial cells (HUVEC) were provided by Dr. Derrick Grant, NIDCR, and human dermal microvascular endothelial (HDME) cells were purchased from Clonetics Corp., San Diego, CA. HUVEC cells were maintained in medium 199E supplemented with 20% FCS, 10 μg/ml heparin, 80 μg/ml endothelial mitogen

(Biomedical Technologies, Inc., Stoughton, MA), glutamine, penicillin, and streptomycin sulfate. HDME cells were maintained in MCDB medium containing glutamine, 5% FCS, 10 ng/ml epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml ascorbic acid, 30 μg/ml heparin, 4 ng/ml FGF2, 4 ng/ml VEGF, 5 ng/ml IGF1, and 50 μg/ml gentamicin.

Cell proliferation was measured using the Cell-Titer colorimetric assay (Promega) as previously described (Vogel et al., 1993). A 100 μl volume of BAE cell suspension at 50,000 cells/ml in DMEM containing 1% FBS and supplemented with 10 ng/ml FGF2 was plated in triplicate in 96 well tissue culture plates either in the presence of peptides in solution or in wells that were pre-coated with 100 μl of the peptides at 4^0 C overnight and blocked with 1% BSA before adding the cells. Cells were grown for 72 hours at 37^0 C in a humidified incubator with 5% CO_2. HUVEC proliferation was measured by the same protocol but using medium 199 containing 5% FCS but without heparin. HDME cell proliferation was measured in MCDB growth medium without heparin, VEGF, or FGF2.

Adhesion

TSP1 and TSP1 peptides in Dulbecco's PBS were adsorbed on bacteriological polystyrene dishes by overnight incubation at 4°. After blocking with 1% BSA in Dulbecco's PBS, adhesion assays were performed by adding cells suspended in DMEM (BAE cells) or medium 199 (human cells) containing 1 mg/ml BSA. Cell attachment and spreading was quantified microscopically. Inhibition assays were performed using the following function blocking antibodies: P1B5 (Gibco-BRL, α3β1), P4C2 (Gibco-BRL, α4β1), and mAb13 (Dr. Ken Yamada, anti-β1). The β1 integrin-activating antibody TS2/16 (Hemler et al., 1984) and the CD98 antibody 4F2 were prepared from hybridomas obtained from the American Type Culture

Collection. Immunofluorescence analysis of cell adhesion was performed on glass substrates as described previously, using BODIPY TR-X phallacidin (Molecular Probes, Inc. Eugene, OR) to visualize F-actin and using murine primary antibodies followed by BODIPY FL anti-mouse IgG to localize integrins, vinculin (Sigma), CD98 (4F2) or phosphotyrosine (PY Plus, Zymed) (Sipes et al., 1999).

Scratch wound repair

The *in vitro* wound healing assay used was a slight modification of that described by Joyce et al. (Joyce et al., 1989). A confluent monolayer of BAE cells pretreated with 10 μg/ml 5-fluorouracil for 24 hours were used in this assay. A straight wound about 2.0 mm wide was made in the monolayers using the flat edge of a sterile cell scraper (Costar #3010), and the cells were allowed to migrate back into the wound site in the presence of TSP1 peptides. Mitosis of the BAE cells in the monolayers was inhibited by addition of 5-fluorouracil, so that the rate of wound closure was due solely to the migration of cells into the wound sites. The distances between the wound margins were measured as soon as the wound was made and 24 hours later using a grid incorporated into the eye piece of the microscope. All data represent the results obtained from three independent scratch wounds for each peptide tested.

CAM angiogenesis assay

Fertilized Leghorn chicken eggs were obtained from Ramona Duck farm (Westminster, CA). At day 3 of development, the embryos were placed on 100 mm petri dishes. Assays were performed as previously described (Iruela-Arispe et al., 1999). Briefly, vitrogen gels containing growth factors (FGF-2 (50ng/gel) and VEGF (250ng/gel)) were allowed to polymerize in the presence or absence of TSP1 peptides. Peptides were filtered on Centricon P100 prior to their

analysis on the CAM assays to eliminate traces of endotoxin. Pellets were applied to the outer 1/3 of the CAM, and the assay was performed for 24h. Detection of capillary growth was done by injection of FITC-dextran in the bloodstream and observation of the pellets under a fluorescent inverted microscope. Positive controls (growth factors and vehicle), as well as negative controls (vehicle alone) were placed in the same CAM and used as reference of 100% stimulation or baseline inhibition (0%), and response to the peptides was determined according to these internal controls. Assays were performed in duplicate in each CAM and in four independent CAMs (total of 8 pellets). Statistical evaluation of the data were performed to check whether groups differ significantly from random by analysis of contingency with Yates' correction.

Results

Adhesion assays were used to determine whether the α3β1 integrin-binding sequence from residues 190-201 of TSP1 (Krutzsch et al., 1999) is recognized by endothelial cells. Endothelial cells attached specifically on immobilized TSP1 peptide 678 but not on the inactive analog peptide 690, in which the essential Arg residue was substituted with an Ala residue (Fig. 1A). Two related peptides with amino acid substitutions that diminished their activity for mediating α3β1-dependent adhesion of breast carcinoma cells (Krutzsch et al., 1999) only weakly supported endothelial cell adhesion (Fig. 1A). All of the peptides had similar capacities for adsorption on the polystyrene substrate used for these assays (2.5 to 3.8 pmoles/mm^2), so the differences in activities of these peptides did not result from differences in their adsorption.

Although some previous publications have reported that TSP1 promotes spreading of

endothelial cells (Morandi et al., 1993; Taraboletti et al., 1990), other investigators have concluded that TSP1 ~~can not~~ promote endothelial cell spreading and disrupts spreading of endothelial cells attached on certain other matrix proteins (Chen et al., 1996; Lahav, 1988; Lawler et al., 1988; Murphy-Ullrich and Höök, 1989). In agreement with the latter reports, bovine aortic endothelial cells harvested from a confluent cobblestone did not spread on TSP1 (Fig. 1B and Fig 2a). However, when a duplicate culture of the same cells was replated at low density to minimize cell-cell contact and harvested at the same time post-feeding, they did (Fig. 1B and Fig 2c). Up-regulation of spreading on TSP1 following loss of cell-cell contact was highly significant ($p < 0.0001$) and specific for TSP1, because spreading on fibronectin and collagen were not induced under the same conditions (Fig 1B and Fig. 2b, d). Sparse cells also displayed a significant increase in spreading on vitronectin ($p = 0.001$), although approximately 60% of the cells harvested from a confluent monolayer also spread on vitronectin, compared to less than 10% on TSP1 (Fig. 1B).

Similar induction of BAE cell spreading following loss of cell-cell contact was observed using the $\alpha 3\beta 1$ integrin-binding peptide from TSP1 (peptide 678, Fig. 3A). Density-dependent spreading on intact TSP1 and the TSP1 peptide were both inhibited by peptide 678 added in solution but were not significantly inhibited by the control peptide 690 (Fig. 2e and Fig. 3A). Inhibition by the active peptide was specific for endothelial cell spreading on TSP1 or the TSP1 peptide, because peptide 678 did not inhibit spreading on fibronectin (Fig. 2f).

Similar density dependence for spreading on TSP1 and the TSP1 peptide 678 was observed with human endothelial cells (Fig 3B). Although only 6% of HDME cells harvested from a confluent monolayer spread following attachment on immobilized TSP1, 29% of those

from a duplicate sparse culture spread on the same substrate. No spreading of the confluent culture was detected on TSP1 peptide 678, but 28% of HDME cells from the sparse culture spread on this peptide. Using HUVEC, sparse cultures showed only a slight increase in spreading (46 ± 7% versus 41 ±5% for confluent cells, $p = 0.36$), but spreading on the peptide 678 was significantly induced (12 ± 3% for sparse cultures versus 3 ± 1% for confluent, $p = 0.008$, data not shown). These data demonstrate that loss of cell-cell contact induces spreading on TSP1 and on its α3β1 integrin-binding peptide for both microvascular and large vessel endothelial cells of bovine and human origins.

The increased spreading of BAE cells on TSP1 is mediated at least in part by α3β1 integrin, because a TSP1 peptide that binds to this integrin (Krutzsch et al., 1999) inhibited spreading on TSP1 by 55% but did not inhibit spreading on fibronectin or vitronectin substrates (Fig. 4A). The αvβ3 integrin also plays some role in BAE cell spreading on TSP1, since the αv integrin antagonist SB223245 partially inhibited spreading on TSP1. The effect of these two inhibitors was additive, producing a 76% inhibition of spreading when combined. Similar results were obtained using the αvβ3 peptide antagonist GRGDSP alone and in combination with peptide 678. Approximately 20% of the spreading response on TSP1 was resistant to the GRGDSP peptide, but combining this peptide with the α3β1 integrin-binding peptide completely inhibited spreading on TSP1.

Primary human large vessel and microvascular endothelial cells also used the α3β1 integrin to mediate spreading on TSP1 (Fig. 4B and results not shown). HUVEC spreading on TSP1 was inhibited 70 ± 7% by peptide 678, whereas spreading on vitronectin was not significantly inhibited (Fig. 4B). Conversely, the αvβ3 antagonist SB223245 completely

inhibited spreading on vitronectin but did not significantly inhibit spreading on TSP1. HDME cell spreading on TSP1 was partially inhibited by the function-blocking integrin antibodies specific for β1 (mAb13) and α3 subunits (P1B5) but not by the α4β1 blocking antibody P4C2 (Fig. 4C), verifying that spreading of these microvascular cells on TSP1 is also mediated by the α3β1 integrin.

The possible involvement of other TSP1 receptors, including αvβ3 integrin, CD36, and heparan sulfate proteoglycans, were further examined using the human endothelial cells. The TSP1-binding integrin αvβ3 did not contribute to adhesion of the human endothelial cells, based on insensitivity to the αv integrin antagonist SB223245 (Fig. 4B and results not shown). Likewise, a function-blocking antibody recognizing the TSP1 receptor CD36 did not block adhesion of HDME cells (Fig. 4C). Of the human endothelial cells used, only HDME cells expressed CD36 as measured by RT-PCR (results not shown). Therefore, expression of CD36 is not required for endothelial cell spreading on TSP1. Heparin also had no effect on spreading of HDME cells on a TSP1 substrate (data not shown). These results demonstrate that the α3β1 integrin contributes to spreading of several types of endothelial cells on TSP1 and are consistent with the previous report that HDME cell adhesion on TSP1 is independent of CD36 and the αvβ3 integrin (Chen et al., 1996).

The α3β1 integrin was localized in lamellipodia of HUVEC cells spreading on TSP1 (Fig. 5a). The β1 integrin-activating protein CD98 showed a similar distribution in cells spreading on TSP1 (Fig. 5b). Both antibodies also labeled the borders of vacuolar structures that formed at the periphery of HUVEC cells spreading on TSP1. These resemble the ring structures reported previously to be induced following treatment of microvascular cells with anti-TSP1 antibodies

(Tolsma et al., 1997). Lamellar spreading on TSP1 was associated with tyrosine phosphorylation at the leading edge of spreading cells (Fig. 5c). Vinculin antibody staining showed no evidence for formation of focal adhesions on TSP1, but some cells showed limited radial organization of vinculin in lamellipodia (Fig. 5d). These structures were not observed in cells stained with the α3β1 integrin antibody and may therefore be induced by another TSP1 receptor, such as the αvβ3 integrin.

Cells spreading on TSP1 peptide 678 also showed organization of α3β1 integrin (Fig. 5e) and CD98 (Fig. 5f) at the cell periphery, supporting their role in mediating spreading on this TSP1 peptide. Vacuolar structures were also observed in lamellipodia spreading on the TSP1 peptide, suggesting that these are induced by engagement of the α3β1 integrin. The spreading observed on glass substrates coated with peptide 678, however, was consistently weaker than that observed using the same peptide adsorbed on polystyrene, so further immunofluorescent analysis of endothelial cells attaching on this peptide was prevented by lack of a suitable substrate.

Based on the localization of CD98 in endothelial cells spreading on TSP1 and its ability to activate β1 integrins (Chandrasekaran et al., 1999; Fenczik et al., 1997), we examined the effect of the CD98 antibody 4F2 on HUVEC spreading on TSP1 (Fig. 6). The CD98 antibody enhanced spreading on TSP1 and peptide 678 to a similar degree as the β1 integrin-activating antibody TS2/16. Stimulation of spreading by both antibodies was specific in that spreading of the treated cells on vitronectin, an αvβ3 integrin ligand, was not affected (Fig. 6).

Interaction of the α3β1 integrin with its ligands can regulate epithelial cell proliferation (Gonzales et al., 1999). We therefore examined the effect of the α3β1 integrin-binding sequence from TSP1 on endothelial cell proliferation. Peptide 678 inhibited BAE cell proliferation in a

dose-dependent manner when added in solution (Fig. 7A). Two control peptides with amino acid substitutions that eliminate integrin binding, 686 and 690 (Krutzsch et al., 1999), did not or only slightly inhibited proliferation of BAE cells (19% inhibition at 100 μM for peptide 690). Similar inhibition by soluble peptide 678 was observed in HUVEC cultures (Fig. 7B). However, plating of HUVEC on immobilized peptide 678 increased their proliferation (Fig. 7B). Similar enhancement of microvascular (HDME) cell proliferation was observed after plating on immobilized peptide 678 (Fig. 7C). Intact TSP1 also had differential effects on endothelial cell proliferation when immobilized versus added in solution. As reported previously for several other types of endothelial cells (Bagavandoss and Wilks, 1990; Panetti et al., 1997; Sheibani and Frazier, 1995; Taraboletti et al., 1990), soluble TSP1 inhibited proliferation of HDME cells stimulated by FGF2, but plating of the same cells on immobilized TSP1 stimulated their proliferation (Fig 7C).

To examine the role of the α3β1 integrin-binding sequence of TSP1 in endothelial cell motility, we determined the effect of peptide 678 on endothelial scratch wound repair (Fig. 8). Cells were arrested using 5-fluorouracil to measure effects on endothelial cell motility in the absence of proliferation. Peptide 678 was a dose-dependent inhibitor of BAE cell migration into the wound. At 30 μM, peptide 678 significantly inhibited endothelial cell migration relative to the control ($p = 0.016$, 2-tailed t-test), and this inhibition was specific in that the inactive analog peptide 690 did not inhibit cell motility in this assay ($p > 0.5$).

The α3β1 integrin recognition sequence in TSP1 may also contribute to angiogenesis, because peptide 678 inhibited angiogenesis in the chick CAM assay ($p<0.005$ at 20 μM, Fig. 9). The dose dependence for inhibition was consistent with the reported IC_{50} of this peptide for

blocking α3β1 integrin-dependent adhesion (Krutzsch et al., 1999) and for inhibiting endothelial cell proliferation *in vitro*. Inhibition of angiogenesis by TSP1 peptide 678 was specific in that substitution of the essential Arg residue with Ala (peptide 690) abolished inhibitory activity in the CAM assay.

Discussion

Although TSP1 is generally recognized as an inhibitor of angiogenesis (Good et al., 1990; Iruela-Arispe et al., 1999), conflicting reports about the effects of TSP1 on endothelial cell adhesion, motility, and proliferation have precluded a clear understanding of the mechanism for its anti-angiogenic activity (BenEzra et al., 1993; Canfield and Schor, 1995; Good et al., 1990; Iruela Arispe et al., 1991; Nicosia and Tuszynski, 1994; Taraboletti et al., 1990). Recognizing that endothelial cells can modulate their expression or activation state of specific TSP1 receptors that transduce opposing signals may lead to a resolution of this conflict. We have demonstrated that sparse endothelial cells recognize an α3β1 integrin-binding sequence in TSP1 that stimulates endothelial cell spreading and proliferation when immobilized on a substratum. Addition of this TSP1 peptide in solution inhibits endothelial cell spreading on TSP1, endothelial cell proliferation and migration *in vitro,* and angiogenesis *in vivo*, presumably by inhibiting TSP1 interactions with this integrin. We also demonstrated that the activity of this integrin to recognize TSP1 is suppressed in a confluent endothelial cell monolayer. Loss of endothelial cell-cell contact during wound repair *in vitro* or angiogenesis *in vivo* could therefore activate this receptor and make endothelial cells responsive to TSP1 signaling through the α3β1 integrin.

The activity of a second TSP1 receptor on endothelial cells that mediates inhibition of

growth factor-stimulated migration, CD36, is regulated by differential expression in endothelial cells from large vessels versus capillaries (Dawson et al., 1997; Swerlick et al., 1992). Thus, CD36-negative endothelial cells with activated α3β1 integrin may recognize TSP1 primarily as an angiogenic signal, whereas CD36-positive endothelial cells with inactive α3β1 integrin would receive only an anti-angiogenic signal (Dawson et al., 1997). Antagonism of FGF2-mediated angiogenic signals by heparin-binding sequences in TSP1 is a second pathway through which TSP1 can inhibit angiogenesis (Iruela-Arispe et al., 1999; Vogel et al., 1993). The responsiveness of this pathway has not been demonstrated to be regulated by endothelial cells. Therefore, endothelial cells receive both pro- and anti-angiogenic signals from TSP1, and the net balance of these signals is controlled by environmental signals that regulate the expression and activity of each TSP1 receptor.

TSP1 expression in endothelial cells is also regulated by cell-cell contact (Canfield et al., 1990; Mumby et al., 1984). Cells without mature cell-cell contacts produce more TSP1 than confluent cells (Mumby et al., 1984). Reports that TSP1 is involved in endothelial cell outgrowth in wound repair assays (Munjal et al., 1990; Vischer et al., 1988), combined with our new data showing that recognition of TSP1 by the α3β1 integrin is activated under the same conditions that stimulate TSP1 production, suggest that coordinate induction of TSP1 expression and activation of its receptor, α3β1 integrin, may stimulate both endothelial cell motility and proliferation during wound repair. This hypothesis is consistent with the pattern of TSP1 expression induced in vascular injury (Reed et al., 1995). Although induction of TSP1 expression during angiogenic responses has been interpreted as a negative feedback pathway to limit angiogenesis (Suzuma et al., 1999), the possibility should be considered that TSP1

immobilized in the extracellular matrix also participates as a positive regulator of neovascularization. This positive signal would be limited, because the α3β1 integrin becomes inactive when endothelial cell-cell contact is established.

Involvement of β1 integrins in endothelial cell adhesion on TSP1 is consistent with several recent studies of TSP1-endothelial cell interactions. Binding of soluble TSP1 to HUVEC was shown to be mediated mostly by heparan sulfate proteoglycans, with some involvement of αvβ3 integrin but not of CD36 (Gupta et al., 1999). However, combinations of these inhibitors could not completely inhibit TSP1 binding to HUVEC, suggesting that additional TSP1 receptors are present on endothelial cells. More relevant to the present studies, HDME cell adhesion on TSP1 was not RGD- or CD36-dependent, and was concluded to be mediated by an undefined TSP1 receptor (Chen et al., 1996). Based on the present data, the α3β1 integrin mediates this adhesive interaction of HDME cells with TSP1.

Previous publications have identified the αvβ3 integrin as a TSP1 receptor on endothelial cells (Gupta et al., 1999; Lawler et al., 1988). We confirmed this result for BAE cells using a specific nonpeptide antagonist of this integrin, and we found that disrupting cell-cell contact somewhat increases the activity of this integrin for mediating BAE cell spreading on TSP1. In contrast, we could not detect a contribution of the αvβ3 integrin on microvascular and large vessel human endothelial cells to their adhesion on TSP1. Rather, α3β1 seems to be the major TSP1-binding integrin on human endothelial cells. On BAE cells, α3β1 integrin also plays a major role in mediating spreading on TSP1 and is strongly induced or activated by loss of cell-cell contact. The other known TSP1-binding integrins, α4β1 and α5β1, do not contribute significantly to this response.

The β1 integrin-activating antibody TS2/16 and a CD98 antibody both increased the activity of the α3β1 integrin, demonstrating that this integrin exists on endothelial cells in a partially inactive state. Although sparse cells deprived of cell-cell contact spread better on TSP1 that confluent cells, both showed similar spreading following activation by TS2/16 (unpublished results), suggesting that differential integrin activation rather than differential expression is responsible. Cell-cell contact may therefore transduce a negative signal that suppresses activity of this integrin. We do not know whether exogenous signals could also regulate activation of the α3β1 integrin on endothelial cells. In breast carcinoma cells, IGF1 and insulin acting through the IGF1 receptor were potent activators of the α3β1 integrin for binding to TSP1 (Chandrasekaran et al., 1999). However, IGF1 and insulin had no significant effect on the activity of α3β1 integrin on endothelial cells (Roberts, unpublished results).

The α3β1 integrin is expressed in confluent endothelial cells, but it is concentrated at the cell-cell junctions in association with the TM4 proteins CD9 and CD151/PETA-3 (Yanez-Mo et al., 1998). In the same study, function blocking antibodies recognizing α3β1 integrin inhibited migration of endothelial cells lacking cell-cell contact. In endothelial cells attaching on TSP1, α3β1 integrin localizes to sites of contact with the substrate. Differential association with integrin-binding components in the cytoplasm or membrane accompanying this redistribution of α3β1 integrin may, therefore, activate the integrin to recognize TSP1 and lead to the induction of the spreading response we observed in cells lacking cell-cell contact.

Several other matrix proteins are known to exert both positive and negative effects on cell proliferation. Altering the architecture of fibronectin (Sechler and Schwarzbauer, 1998) or type I collagen matrices (Koyama et al., 1996) can reverse their effects on cell cycle progression.

Differential expression of integrins can reverse the effects of laminins and tenascin on cell proliferation (Mainiero et al., 1997; Yokosaki et al., 1996). TSP1, likewise, expresses both pro- and anti-proliferative activities for specific cell types, but its activity toward endothelial cells has been generally regarded as anti-proliferative (Bagavandoss and Wilks, 1990; Taraboletti et al., 1990). However, we have now demonstrated that interaction with immobilized intact TSP1 or the TSP1 peptide 678 through the endothelial α3β1 integrin stimulates proliferation of endothelial cells. Binding of laminin-5 to the α3β1 integrin was recently demonstrated to stimulate proliferation of mammary epithelial cells (Gonzales et al., 1999), suggesting that the growth promoting activity of immobilized TSP1 for endothelial cells may be a general response to α3β1 ligand binding. Since addition of soluble TSP1 peptide that is recognized by this integrin also inhibits endothelial cell motility in the absence of proliferation, α3β1 integrin interaction with intact immobilized TSP1 may stimulate both endothelial cell proliferation and motility. Defining the specific sequences in TSP1 and the respective endothelial cell receptors that are responsible for both its pro- and anti-angiogenic activities may allow us to isolate each activity and lead to development of peptides, gene therapy approaches, or small molecule analogs of TSP1 with more specific anti-angiogenic activities.

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Footnotes

Abbreviations: BAE, bovine aortic endothelial; HDME, human dermal microvascular endothelial; HUVEC, human umbilical vein endothelial cells; peptide 678, FQGVLQNVRFVF; TSP1, human thrombospondin-1

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Figure legends

Fig. 1. Adhesion of endothelial cells on an α3β1 integrin-binding peptide from TSP1. Panel A: TSP1 peptide 678 (FQGVLQNVRFVF) or analogs of this peptide with the indicated Ala substitutions (*) were adsorbed on bacteriological polystyrene substrates at 10 μM in PBS. Direct adhesion of BAE cells to the adsorbed peptides or uncoated substrate (control) are presented as mean ± SD, n = 3. Panel B: Loss of cell-cell contact stimulates endothelial cell spreading on TSP1. Two flasks of BAE cells were grown to confluence. One flask was harvested and replated in fresh medium at 25% confluency. Fresh medium was added at the same time to the second flask. After 16 h, cells from both flasks were dissociated using EDTA, and adhesion was measured on substrates coated with 40 μg/ml TSP1, 10 μg/ml vitronectin, 20 μg/ml plasma fibronectin, or 5 μg/ml type I collagen. The percent spread cells from confluent (solid bars) or sparse cultures (striped bars) after 60 min is presented as mean ± SD, n = 3 for a representative experiment.

Fig. 2. Spreading on TSP1 induced by loss of cell-cell contact is inhibited by the α3β1 integrin-binding peptide from TSP1. BAE cells from confluent (a, b) or sparse cultures (c-f) were incubated for 60 min on substrates coated with 40 μg/ml TSP1 (a, c, e) or 20 μg/ml fibronectin (b, d, f). Inhibition by 30 μM TSP1 peptide 678 is presented in (e-f). Cells were fixed with 1% glutaraldehyde and stained using Diff-quik. Bar in panel a = 25 μm.

Fig. 3. Loss of cell-cell contact induces endothelial cell spreading on TSP1 peptide 678. Panel A: Adhesion of sparse or confluent BAE cells to substrates coated with 40 μg/ml TSP1 (solid

bars) or 10 μM TSP1 peptide 678 (striped bars) was determined as in Fig. 1B. Spreading was determined microscopically for cells with no additions, in the presence of 10 μM peptide 678, or in the presence of 30 μM of the control peptide 690. Results are presented as mean ± SD, n = 3. Panel B: HDME cells harvested from confluent or sparse cultures as in Fig. 1 were plated on substrates coated with TSP1 (solid bars), peptide 678 (striped bars), or type I collagen (open bars). The percent spread cells was determined at 60 min.

Fig. 4. Both α3β1 and αvβ3 integrins mediate spreading of endothelial cells on thrombospondin-1. Panel A: BAE cell adhesion to TSP1 (solid bars), vitronectin (striped bars), or plasma fibronectin (open bars) was measured in the presence of 30 μM TSP1 peptide 678, 1 μM of the αvβ3 integrin antagonist SB223245, 300 μM of the integrin antagonist peptide GRGDSP, or the indicated combinations. Results are expressed as percent of the response for untreated cells, mean ± S.D., n = 3. Panel B: HUVEC spreading on substrates coated with TSP1 (solid bars) or vitronectin (striped bars) was determined in the presence of 20 μM peptide 678, 1 μM αIIbβ3 antagonist SB208651, 1 μM αvβ3 antagonist SB223245, or 20 μM peptide 678 plus 1 μM SB223245. Spreading is presented as a percent of the respective controls without inhibitors (31 cells/mm^2 for TSP1 and 10 cells/mm^2 for vitronectin). Panel C: Inhibition of HDME cell spreading on TSP1 (solid bars) or type I collagen (striped bars) was determined in the presence of the indicated function blocking antibodies at 5 μg/ml: anti-CD36 (OKM5), anti-integrin β1 (mAb13), anti-integrin α3 (P1B5), and anti-integrin α4 (P4C2).

Fig. 5. Integrin and CD98 localization in endothelial cells spreading on TSP1 or TSP1 peptide

678 substrates. HUVEC attached on TSP1 (panels a-d) or TSP1 peptide 678 (panels e, f) were stained using antibodies to α3β1 integrin (panels a, e), CD98 (panels b, f), phosphotyrosine (panel c), or vinculin (panel d). Bar in panel a = 25 μm.

Fig. 6. β1 Integrin- and CD98-activating antibodies induce HUVEC spreading on TSP1 and TSP1 peptide 678. Untreated HUVEC (control) or cells in the presence of 5 μg/ml of the β1 integrin activating antibody (TS2/16) or CD98 antibody (4F2) were incubated on substrates coated with 40 μg/ml TSP1 (solid bars), 5 μM peptide 678 (striped bars), or 5 μg/ml vitronectin (open bars). Cell spreading is expressed as a percent of the response for untreated cells, mean ± S.D., n = 3.

Fig. 7. Modulation of endothelial cell proliferation by an α3β1 integrin binding peptide from TSP1. Panel A: Proliferation of BAE cells was assayed in the presence of the indicated concentrations of TSP1 peptide 678 (FQGVLQNVRFVF, ●) or the control peptides 686 (FQGVLQAVRFVF, ▲), and 690 (FQGVLQNVAFVF, O). Briefly, 100 μl of a $5x10^4$ cell/ml suspension of BAE cells were seeded in triplicate into 96 well tissue culture plate in DMEM medium containing 1% FCS, 10 ng/ml of FGF and peptides at 1-40 μM concentrations. Cells were incubated for 72 h, and proliferation was measured using the Celltiter tetrazolium assay (Promega). Panel B: HUVEC proliferation was determined in the presence of the indicated concentrations of TSP1 peptide 678 immobilized on the substrate (solid bars) or added in solution (striped bars). Panel C: HDME cell proliferation was determined in the presence of 10 ng/ml FGF2 and the indicated concentrations of TSP1 added in the medium (△) or immobilized

on the substrate (●) or in wells coated with the indicated concentrations of peptide 678 (▲). Results are presented as mean ± S.D. and are normalized to controls without TSP1 or peptide.

Fig. 8. TSP1 peptide 678 inhibits wound healing of BAE cells. BAE cells were seeded at a density of $2x10^5$ cells/well of 6 well tissue culture plates in complete growth medium supplemented with 10% FBS. After the cells formed a confluent cobblestone, cells were arrested using 10 μg/ml 5-fluorouracil for 48 h. Scrape wounds of 2 mm width were made in the wells, and the cells were further incubated with medium containing 10% FBS, 10 μg/ml 5 fluorouracil and peptides 686 (solid bars) or 690 (striped bars). Measurements of the distance between the wound margins were taken at 0 and 24 h, and the net migration is expressed as mean ± SEM for triplicates.

Fig. 9. TSP1 peptide 678 inhibits angiogenesis. Polymerized collagen gels containing angiogenic growth factors in the presence or absence of the indicated concentrations of the TSP1 peptide FQGVLQNVRFVF (peptide 678, solid bars) or the inactive analog FQGVLQNVAFVF (peptide 690, striped bars) were placed on the outer 1/3 of 10 d CAMs for 24 h. Each CAM contained two pellets for each peptide concentration, as well as, positive and negative controls. Ability of the peptides to modulate growth-factor driven angiogenesis was assessed by injection of FITC dextran. The percent inhibition relative to controls is presented as mean ± SD for each group (n = 8).

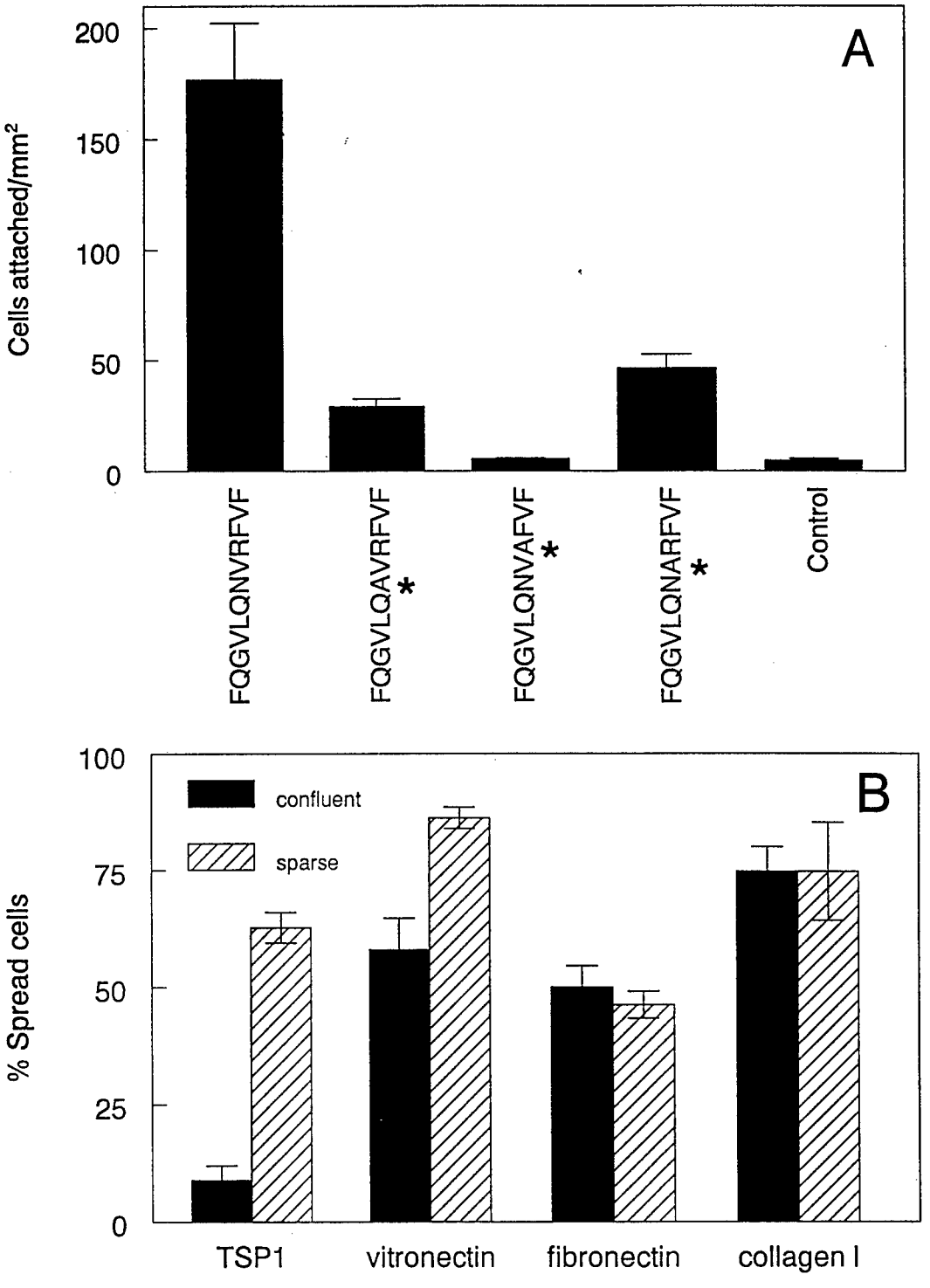

A
Cells attached/mm²
200
150
100
50
0
FQGVLQNVRRFVF
FQGVLQAVRRFVF
*
FQGVLQNVAFVF
*
FQGVLQNARFVF
*
Control
B
% Spread cells
100
75
50
25
0
confluent
sparse
TSP1
vitronectin
fibronectin
collagen I

FIGURE 1

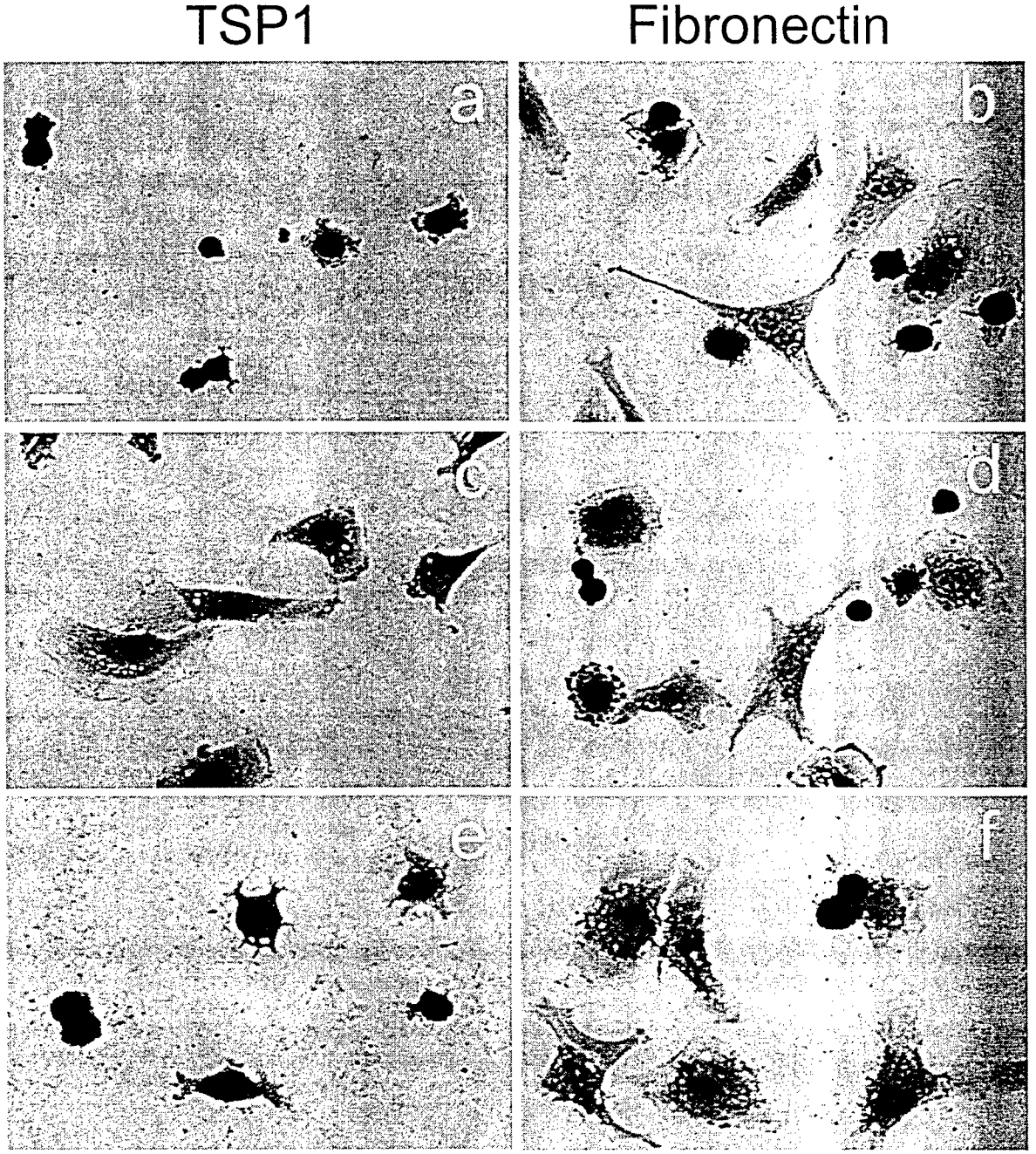
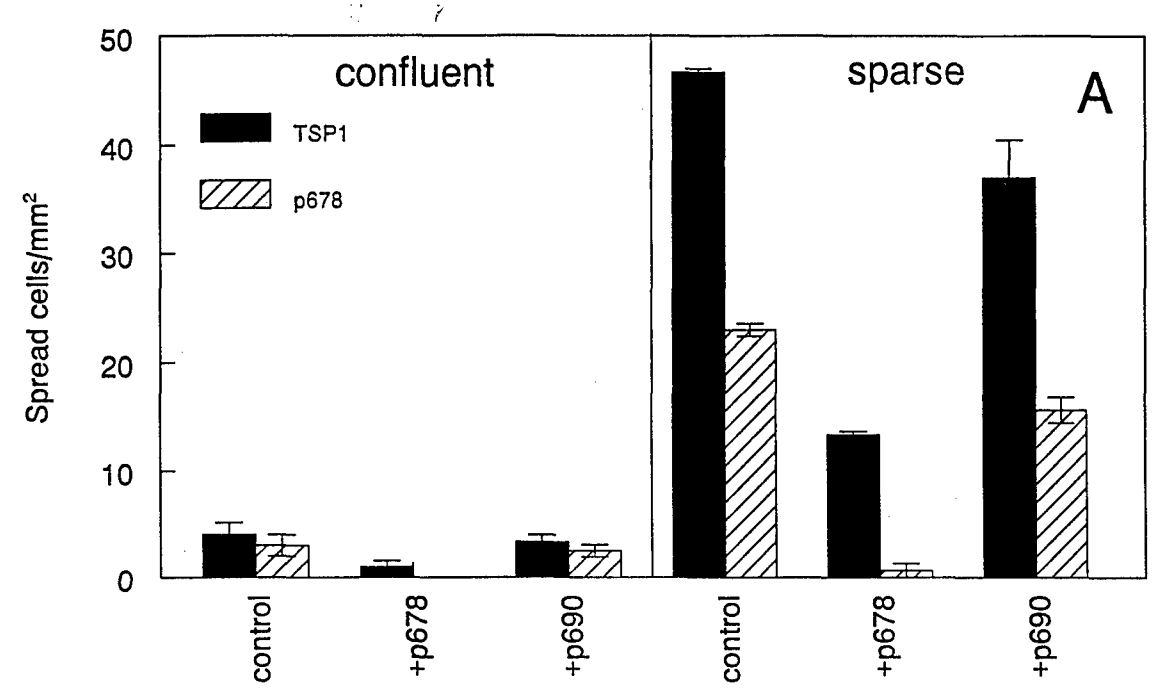


FIGURE 2


confluent
sparse
A
TSP1
p678
Spread cells/mm²
50
40
30
20
10
0
control
+p678
+p690
control
+p678
+p690

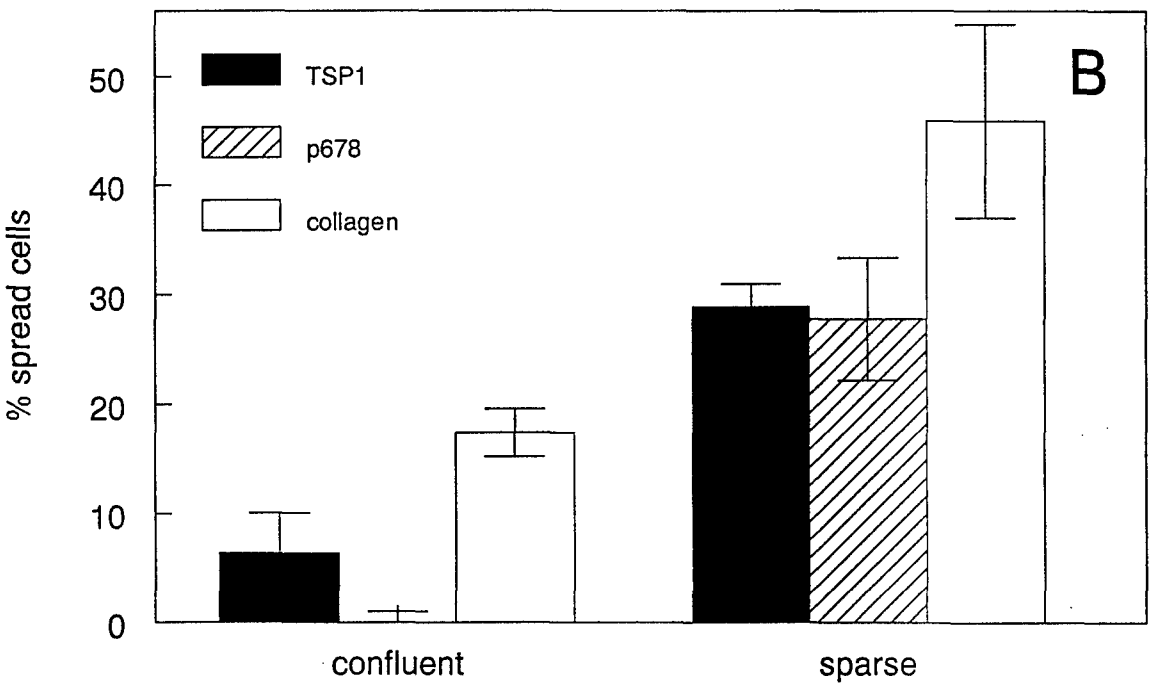
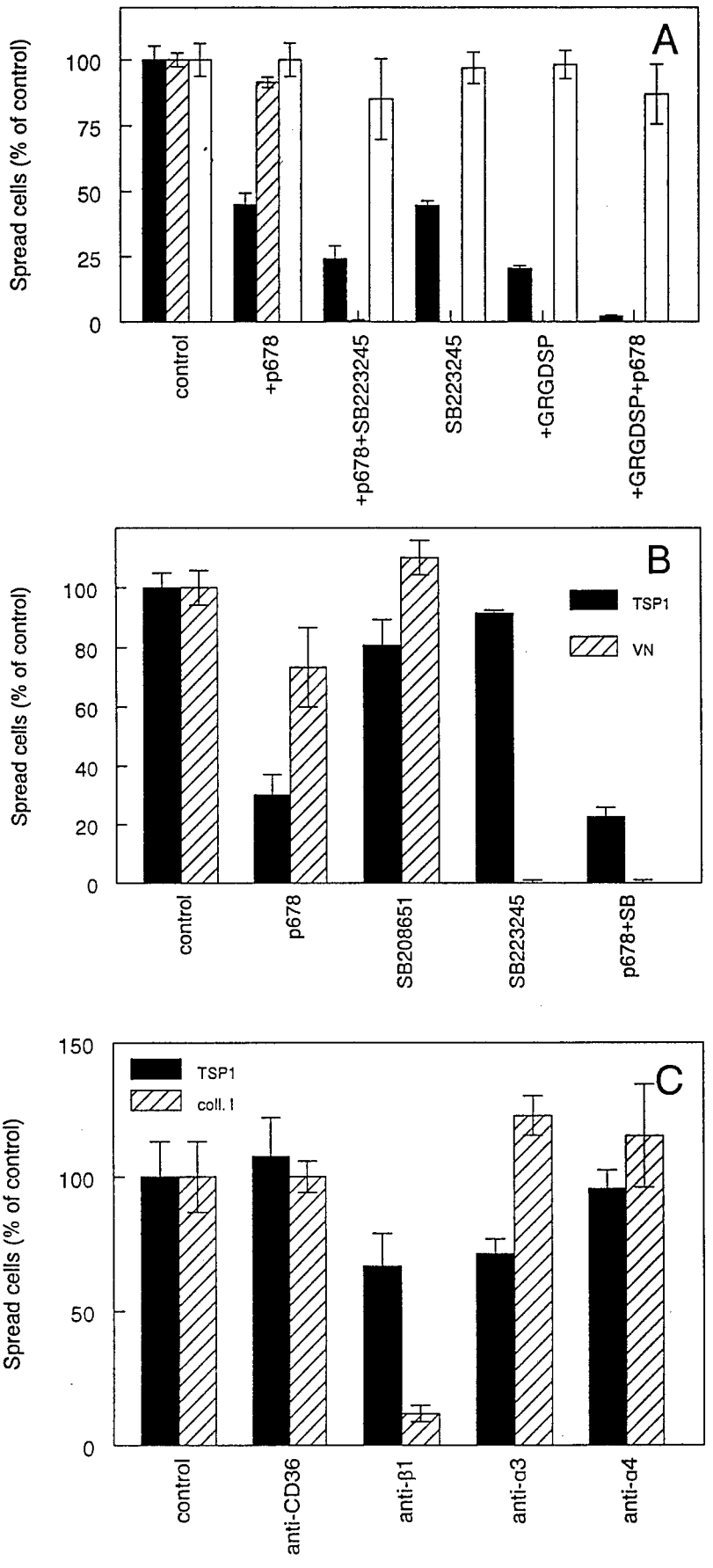

B
TSP1
p678
collagen
% spread cells
50
40
30
20
10
0
confluent
sparse

FIGURE 3

FIGURE 4


A
Spread cells (% of control)
100
75
50
25
0
control
+p678
+p678+SB223245
SB223245
+GRGDSP
+GRGDSP+p678
B
Spread cells (% of control)
100
80
60
40
20
0
TSP1
VN
control
p678
SB208651
SB223245
p678+SB
C
Spread cells (% of control)
150
100
50
0
TSP1
coll. I
control
anti-CD36
anti-β1
anti-α3
anti-α4

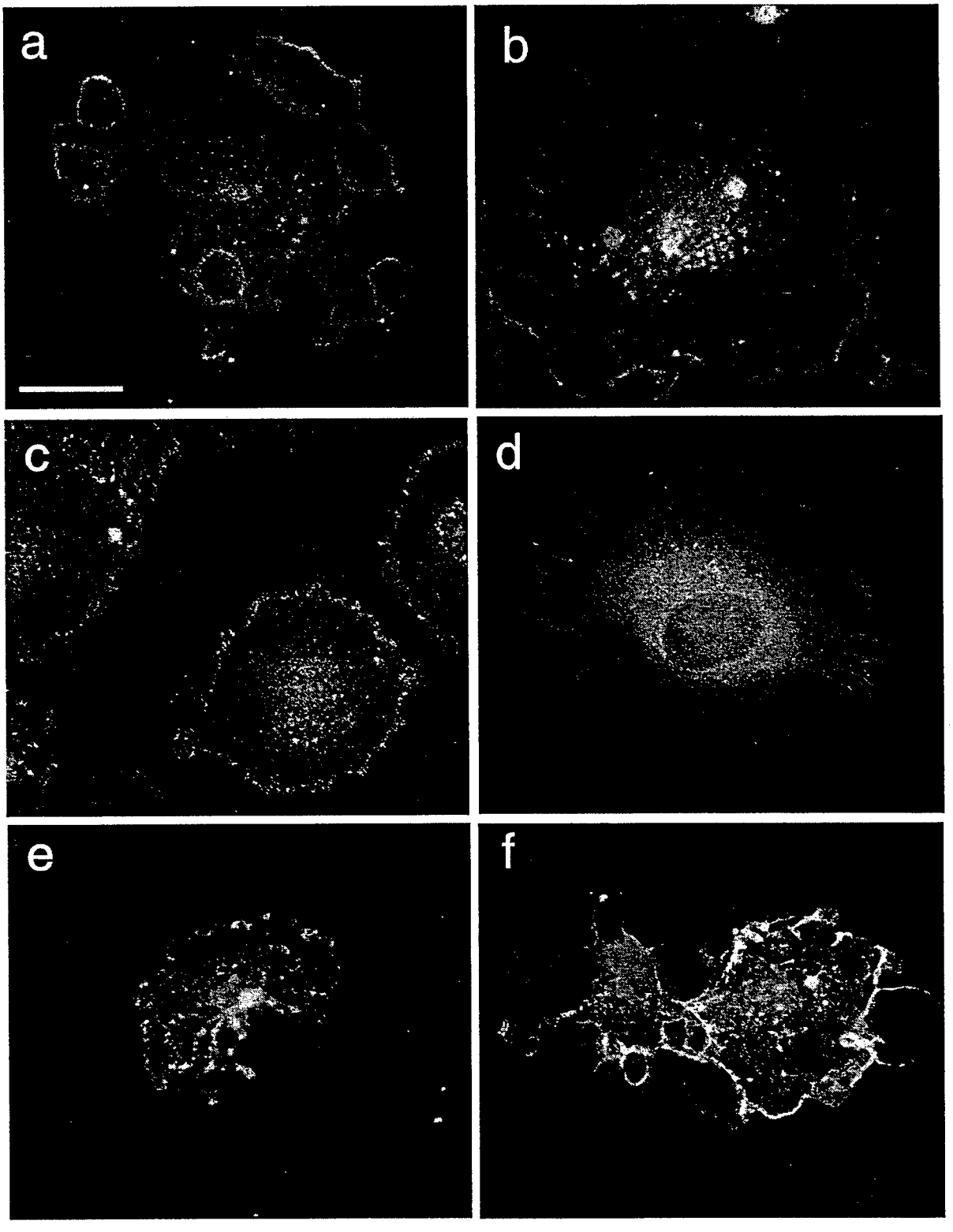


FIGURE 5

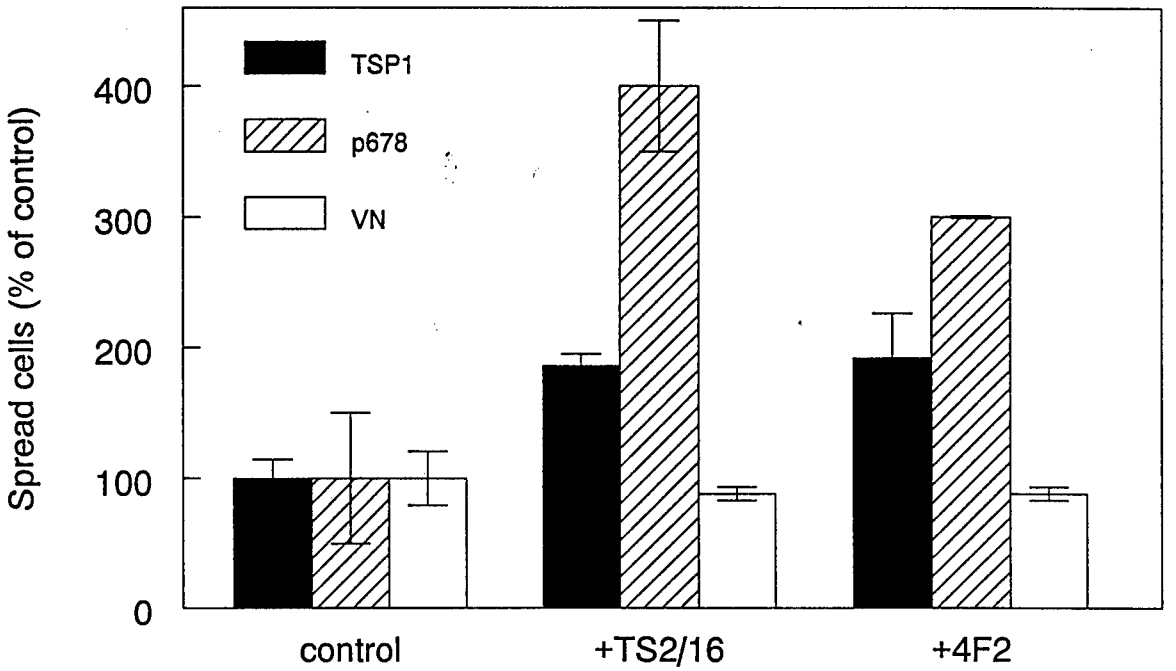
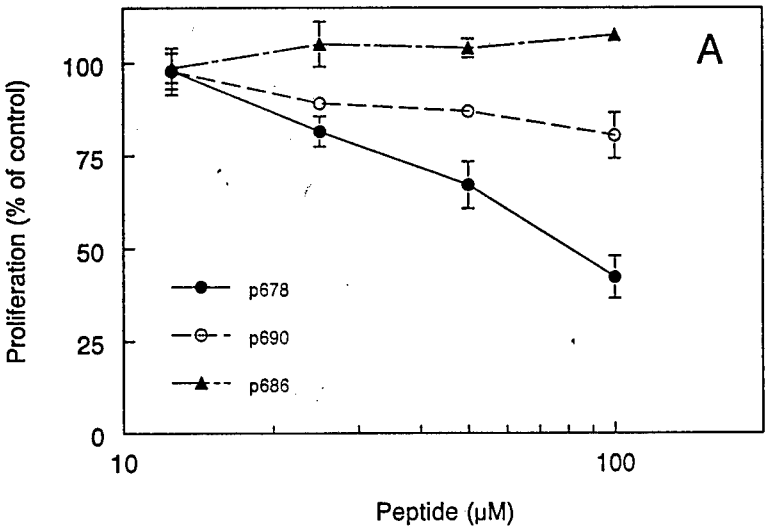
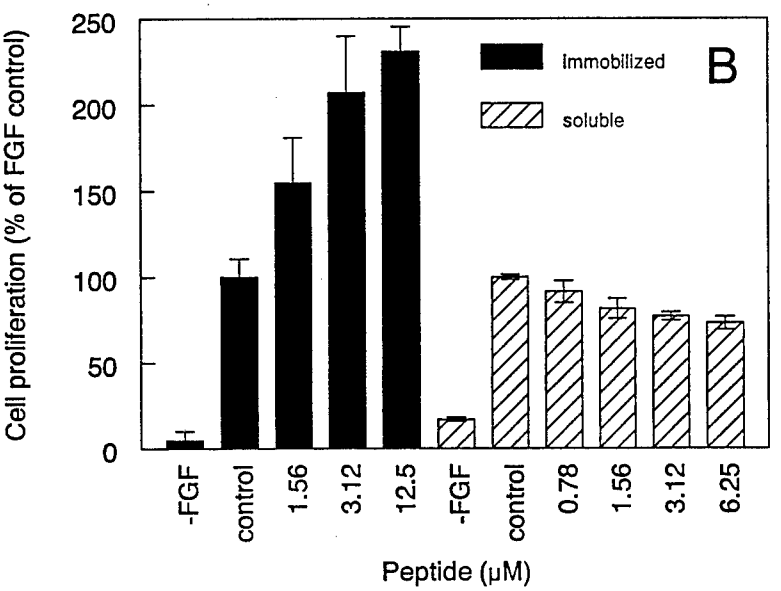

TSP1
p678
VN
Spread cells (% of control)
400
300
200
100
0
control
+TS2/16
+4F2

FIGURE 6


A
Proliferation (% of control)
100
75
50
25
0
p678
p690
p686
10
100
Peptide (μM)


B
Cell proliferation (% of FGF control)
250
200
150
100
50
0
Immobilized
soluble
-FGF
control
1.56
3.12
12.5
-FGF
control
0.78
1.56
3.12
6.25
Peptide (μM)

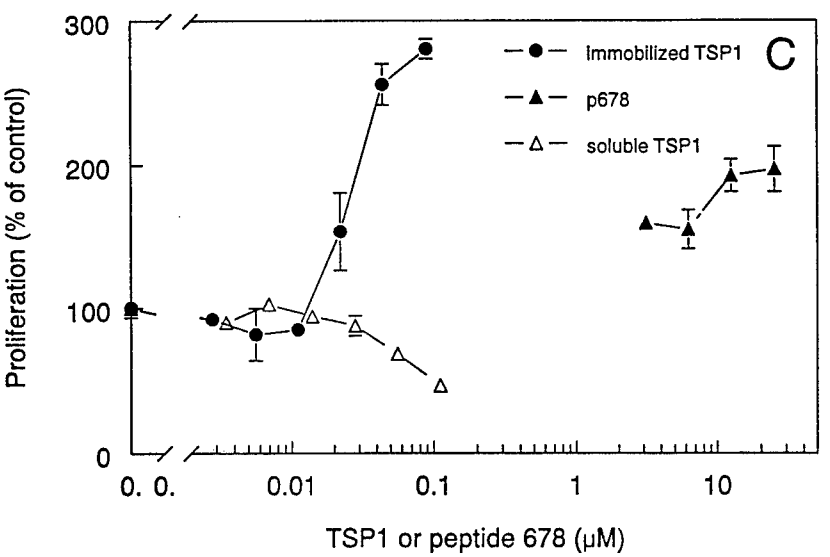

C
Proliferation (% of control)
300
200
100
0
Immobilized TSP1
p678
soluble TSP1
0. 0.
0.01
0.1
1
10
TSP1 or peptide 678 (μM)

FIGURE 7

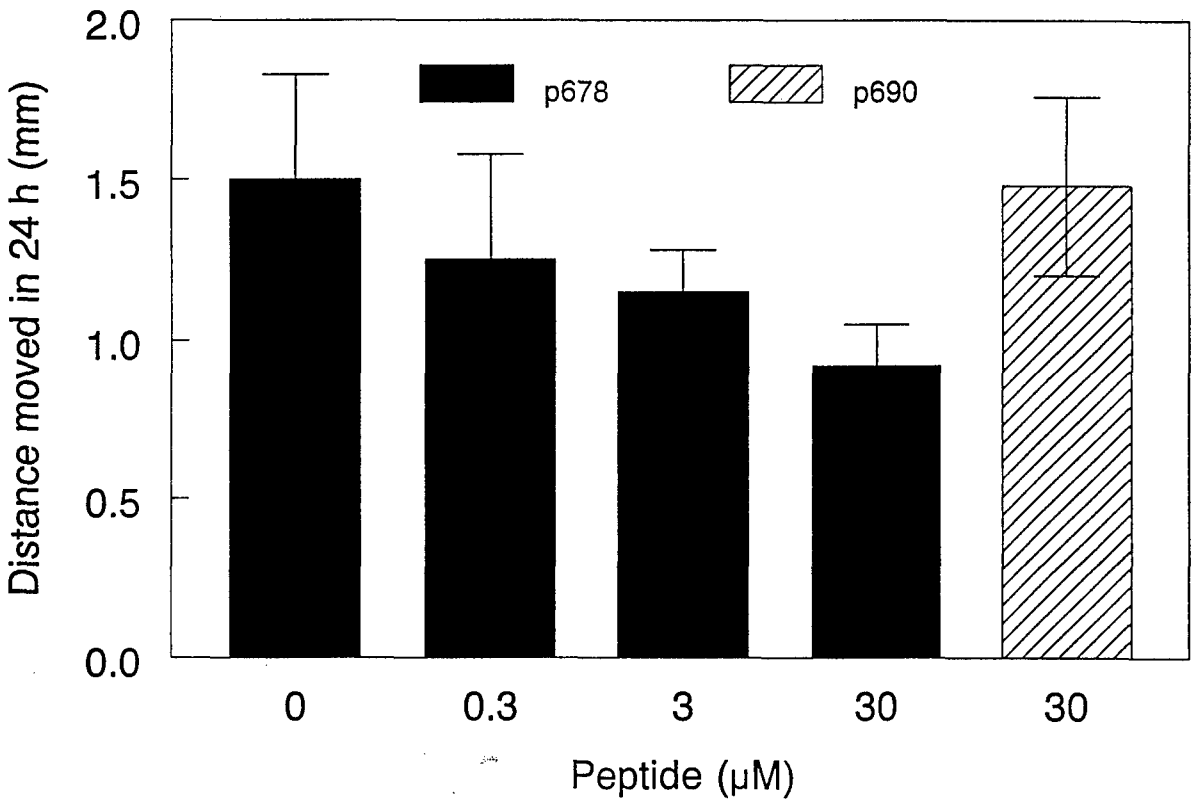

2.0
1.5
1.0
0.5
0.0
Distance moved in 24 h (mm)
p678
p690
0
0.3
3
30
30
Peptide (μM)

FIGURE 8

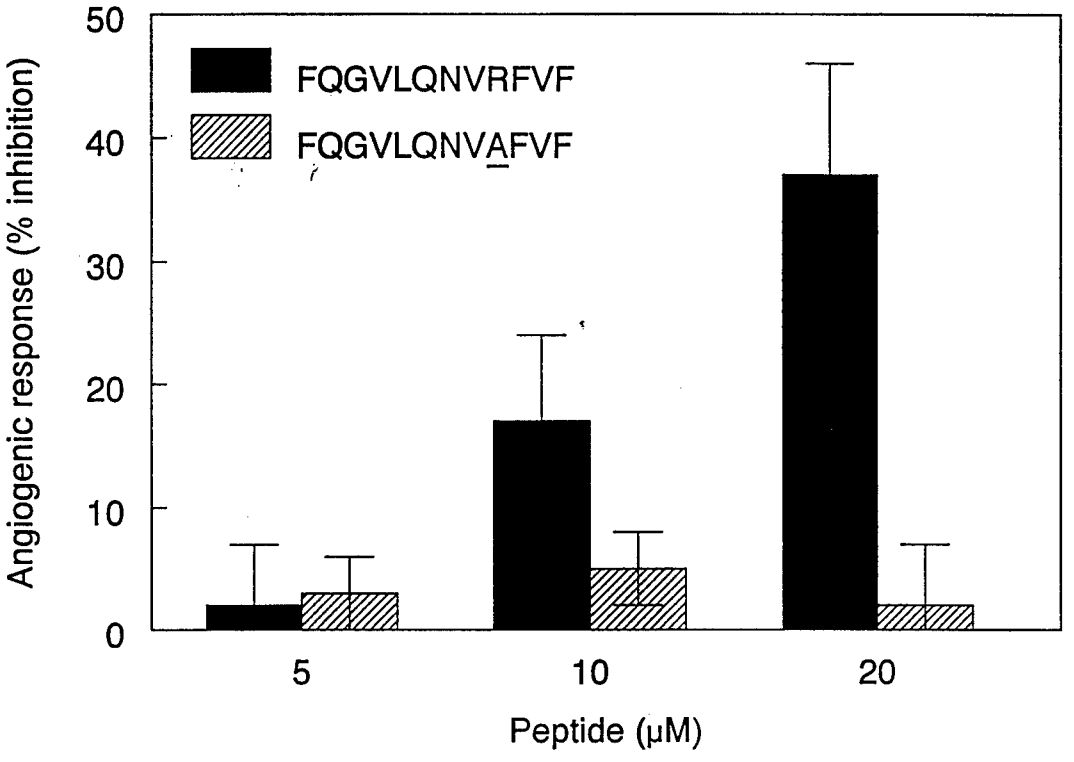

50
40
30
20
10
0
Angiogenic response (% inhibition)
FQGVLQNVRFVF
FQGVLQNVAFVF
5
10
20
Peptide (μM)

FIGURE 9

T12

THROMBOSPONDIN TYPE I REPEAT PEPTIDES SPECIFICALLY INDUCE APOPTOSIS OF ENDOTHELIAL CELLS Neng-hua Guo, Henry C Krutzsch, John K. Inman, and David D. Roberts, Laboratory of Pathology, NCI, and NIAID, NIH, Bethesda, MD 20892-1500

Thrombospondin-1 (TSP) is an inhibitor of angiogenesis that modulates endothelial cell adhesion, motility, and growth. The anti-proliferative activity of TSP is mimicked by synthetic peptides derived from the type I repeats of TSP that antagonize FGF-2 and activate latent TGFβ. Decreases in cell number and morphological changes induced by these peptides suggested that these TSP analogs may induce programmed cell death. Ficoll conjugates containing peptides from the type I repeats of TSP induced DNA fragmentation and inter-nucleosomal cleavage in bovine aortic endothelial cells. The response was specific to the endothelial cells, as no DNA fragmentation was induced in a breast carcinoma cell line, even though TSP and the peptide conjugates inhibited growth of both cell types. Apoptosis did not depend on activation of latent TGFβ, as peptides lacking the activating sequence KRFK were also active. Induction of programmed endothelial cell death by the peptides was decreased when endothelial cell cultures reached confluence. The activity of the peptides could also be inhibited by growth of the cells on fibronectin substrates or in the presence of the phosphatase inhibitor sodium vanadate. These results demonstrate that induction of apoptosis by the TSP analogs is not a general cytotoxic effect and is dependent on a lack of survival-promoting signals such as those provided by a fibronectin matrix. The anti-tumor activities of TSP and these TSP analogs may therefore result from the selective sensitivity of endothelial cells on provisional matrix in newly formed blood vessels to induction of apoptosis.

Presented in "The TSP Gene-Family & its Functional Relatives" meeting, June 19-22,1996 Univ. of Wash. Seattle.

DAMD17-94-J-4499

Appendix I (pp 133-134)

ROLE OF TYPE I REPEATS IN INHIBITION OF BREAST CARCINOMA GROWTH BY THROMBOSPONDIN-1

David D. Roberts, Neng-hua Guo, Lakshmi Chandrasekaran, S. Chandrasekaran, John K. Inman, and Henry C. Krutzsch

Laboratory of Pathology, National Cancer Institute, and Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892-1500

Thrombospondin-1 (TSP1) is an extracellular matrix protein that modulates endothelial cell responses to growth factors and inhibits angiogenesis. Over-expression of TSP1 in breast carcinoma cells suppresses tumor growth and metastasis in mouse xenografts. To define the role of the type I repeats of TSP1 in these activities, we have examined the activities of synthetic peptide analogs based on these repeats and prepared TSP1 expression vectors with site-directed mutations in these repeats.

Synthetic peptides from the type I repeats inhibit endothelial cell growth. The peptides antagonize endothelial cell responses to basic fibroblast growth factor and activate latent transforming growth factor β (TGFβ). We have now shown that the inhibitory activity of the peptides for endothelial cells is independent of their ability to activate latent transforming growth factor β and can be reproduced by D-reverse peptide analogs.

To define the structural basis for the anti-angiogenic activities of these peptides, we prepared analogs of the TSP1 peptide KRFKQDGGWSHWSPWSSC. L-forward, L-reverse, and D-reverse (retro-inverso) analogs displayed identical activities for binding to heparin, demonstrating a lack of stereospecificity for heparin binding. The L-reverse, and D-reverse peptides, however, had somewhat decreased abilities to activate latent TGFβ. Conjugation of the forward peptides through a C-terminal thioether and the reverse peptides through an N-terminal thioether to polysucrose abolished the adhesive activity of the peptides and enhanced their anti-proliferative activities for endothelial and breast

Keywords: **endothelial cells, extracellular matrix, angiogenesis, proliferation, mutagenesis**

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Presented in the DOD Breast Cancer Research Program 'Era Of Hope' meeting, Oct.31-Nov.4, 1997, Washington D.C.

carcinoma cells stimulated by basic fibroblast growth factor. Their anti-proliferative activity was independent of latent TGFβ activation, because substitution of an Ala residue for the essential Phe residue in the TSP1 type-I repeat peptide increased its potency for inhibiting TSP1 binding to heparin and for inhibiting endothelial cell proliferation. Although the conjugated peptides were inactive *in vivo*, an unconjugated retro-inverso analog of the native TSP peptide inhibited breast tumor growth in a mouse xenograft model. Thus, these TSP-derived peptide analogs antagonize endothelial growth through their heparin-binding activity rather than through activation of latent TGFβ or increasing cell adhesion.

These TSP1 analogs induced programmed cell death in bovine aortic endothelial cells based on morphological changes, assessment of DNA fragmentation, and inter-nucleosomal DNA cleavage. Intact TSP1 also induced DNA fragmentation. The endothelial cell response was specific, as no DNA fragmentation was induced in MDA435 breast carcinoma cells, even though TSP1 and the peptide conjugates inhibited growth of both cell types. Apoptosis did not depend on activation of latent TGFβ by the activating sequence RFK, as peptides lacking this sequence also induced apoptosis. Apoptosis was not sensitive to inhibitors of ceramide generation but was inhibited by the phosphatase inhibitor vanadate. Induction of DNA fragmentation by the peptides was decreased when endothelial cell cultures reached confluence. Growth of the cells on a fibronectin substrate also suppressed induction of apoptosis by TSP1 or the peptides. Differential sensitivities to kinase inhibitors suggest that apoptosis and inhibition of proliferation are mediated by distinct signal transduction pathways. These results demonstrate that induction of apoptosis by the TSP1 analogs is not a general cytotoxic effect and is conditional on a lack of strong survival-promoting signals, such as those provided by a fibronectin matrix.

The role of the TGFβ-activating and fibroblast growth factor-antagonist type I repeat sequences in this activity was examined using site-directed mutagenesis of the Phe and Trp residues required for activity of peptides derived from TSP1. Stably transfected breast carcinoma cell lines expressing full length TSP1 with a Trp(441)Ala or a Phe(432)Ala mutation in the second type I repeat were characterized for tumorigenic potential *in vivo* and behavior *in vitro*. Three transfectant clones of MDA MB435 with high levels of mutant TSP expression were injected orthotopically in the mammary fat pads of athymic mice. Clones from TSP1 (W441A) transfection produced tumors with the same or larger tumor masses than controls, whereas those from THBS F432A clones were smaller than controls.

We conclude that stable analogs of active peptides from TSP1 may be useful as therapeutic inhibitors of angiogenesis stimulated by basic fibroblast growth factor. These stable peptide analogs inhibit breast cancer growth in mouse xenografts. The anti-tumor activity of TSP1 and these peptides may result from an increased sensitivity to apoptosis in endothelial cells adjacent to a provisional matrix during formation of new vascular beds in tumors.

DAMD17-94-J-4499

Appendix J (pp 135)

1728

IDENTIFICATION OF P62, PHOSPHOTYROSINE-INDEPENDENT LI[GAND] DOMAIN OF P56[LCK], AS A NOVEL INTEGRIN ASSOCIATED PROTE[IN]

K.J. Askins, C. Xia, and D. Sheppard)) Lung Biology Center and Department of Medicine, University of California, San Francisco, San Francisco General Hospital, 1001 Potrero Ave., San Francisco, CA 94110.

The integrin αvβ6 is restricted to epithelial cells and its' expression is upregulated during development, tissue injury and tumorigenesis. The cytoplasmic domain of the β6 subunit contains both regions which are conserved among the different β subunits and a unique 11 amino acid extension which we have previously shown to be involved in β6-mediated cell proliferation. To further study the function of this integrin we have carried out a yeast two-hybrid screen using the cytoplasmic domain of the β6 subunit as a bait. One of the interacting proteins we identified is p62, the phosphotyrosine –independent ligand for the SH2 domain of p56[lck], a cytosolic protein which also interacts with the atypical protein kinase C isoforms ζ and λ. The region of β6 which interacts with p62 did not lie within the unique 11aa extension but did include the two tyrosine containing motifs conserved in many integrin β subunits. Consistent with this result we also found that p62 could interact with the homologous region of the β1, β2 and β3 cytoplasmic domains in the yeast two-hybrid assay. In vitro binding studies showed that a GST-β6 fusion protein could bind to Myc-tagged p62 expressed in β6 transfected 293 cells. These results suggest that p62 may play a role in modulating cellular responses to integrins.

1729

...SARLAF ELICITS INTEGRIN $\alpha_{IIb}\beta_3$ DEPENDENT ...VATION WHICH IS INDEPENDENT OF FIBRINOGEN BINDING.

((JM Derrick, M Poncz* and TK Gartner†)) Dept. of Pharmacology, University of Tennessee, Memphis, TN 38163, *Childrens Hospital of Phildelphia, PA, and †Dept. of Microbiology and Molecular Cell Sciences, University of Memphis, Memphis, TN.

LSARLAF (LSA), the only integrin dependent peptide agonist characterized to date, binds to the fibrinogen (Fg) receptor on platelets (the integrin $\alpha_{IIb}\beta_3$), and causes platelets to agglutinate and secrete their α-granule contents. Biochemical tests demonstrated that LSA-induced platelet agglutination and secretion are dependent on $\alpha_{IIb}\beta_3$, but not on receptor crosslinking by Fg. Use of a kinase inhibitor showed that LSA-induced platelet agglutination is not secretion dependent and that LSA-induced platelet secretion is dependent on integrin mediated signal transduction. Thrombin receptor activating peptide caused secretion from normal and Type I thrombasthenic platelets (nearly no surface $\alpha_{IIb}\beta_3$) and the Type II thrombasthenic platelets (a single amino acid substitution and only 20-30% of normal $\alpha_{IIb}\beta_3$ on their surface). In contrast, LSA caused secretion of from only normal platelets, and Type II thrombasthenic platelets. The fact that LSA did not induce secretion by the Type I platelets demonstrates unequivocally that LSA induced secretion is $\alpha_{IIb}\beta_3$ dependent. The ability of LSA to stimulate secretion from the Type II platelets even though the altered receptors can not bind Fg confirms the observation that LSA induced signaling is not dependent on Fg binding. Thus, LSA-induced platelet agglutination may be sufficient to cause signal transduction and platelet activation.

1730

MAPPING THE BINDING SITE FOR FIBRINOGEN WITHIN THE α_MI-DOMAIN OF INTEGRIN $\alpha_M\beta_2$.

((Tatiana P. Ugarova, Dmitry A. Solovjov, Li Zhang and Edward F. Plow)) J. J. Jacobs Center for Thrombosis and Vascular Biology, Cleveland Clinic Foundation, Cleveland, OH, 44195

Leukocyte integrin $\alpha_M\beta_2$ interacts with fibrinogen during the immune-inflammatory response. The binding site for $\alpha_M\beta_2$ resides within the peripheral D domain of fibrinogen (Fg). We have recently shown that peptide P2, γ377-395, originating from the COOH-terminal part of the γ-chain of Fg, is a potent inhibitor of the $\alpha_M\beta_2$-mediated adhesion. The active determinant in P2 was localized to γ383-395. P2 specifically bound to the recombinant α_MI-domain of the receptor, suggesting that P2, together with the previously identified sequence γ190-202 (P1), may compose the binding site for $\alpha_M\beta_2$ within Fg. Using a series of 293 cells expressing mutant forms of the α_MI-domain in the context of the $\alpha_M\beta_2$ heterodimer, we have tested adhesion of these cells to P2 and P1. In these mutants, the segments within the α_MI-domain were switched to the homologous sequences of $\alpha_L\beta_2$, a receptor without Fg-binding function. Many mutants were functional, whereas several mutants failed to support adhesion. The segments within the α_MI-domain required for binding were: P^{147}-R^{152}, P^{201}-K^{217}, K^{245}FG and E^{253}-R^{261}. The localized P2-P1-binding site revealed similarity to the previously mapped NIF(neutrophil inhibitory factor) recognition site. This finding accounts for the ability of NIF to inhibit the $\alpha_M\beta_2$-mediated adhesion to Fg.

Presented in the 38th American Society for Cell Biology Annual Meeting, December 12-16, 1998 San Francisco, CA

Extracellular Matrix and Cell Signaling I (1731-1732).

1731

Two Distinct Phases in Integrin-Mediated Raf Activation

Alan Howe and R.L. Juliano. Dept. of Pharmacology and the Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill

We have eaxmined the role of Ras-Raf interaction, Raf membrane localization, and PKC activity in integrin-mediated activation of Raf. Time course experiments involving transfection of epitope-tagged wild type Raf (Raf-WT) or a Raf point mutant deficient in Ras binding (Raf-R89L), as well as pharmacological inhibition of PKC activity demonstrated that integrin-mediated activation of Raf occurs in two phases. Specifically, efficient early activation of Raf required Raf-Ras interaction, but was not affected by inhibitors of PKC activity, while a lower, sustained level of activity was independent of Raf-Ras interaction, but was reduced by PKC inhibitors. The combination of PKC inhibition and lack of Ras binding completely blocked integrin-mediated Raf activation. Interestingly, the activity of a membrane-targeted form of the Raf point mutant (Raf-R89L-CAAX) was also regulated by adhesion. Specifically, Raf-R89L-CAAX acitivty was low in non-adherent cells, was rapidly stimulated to wild type levels by adhesion to fibronectin, and remained at near-maximal levels longer than Raf-WT activity. The activation of all three Raf proteins was ablated by cytochalasin D, demonstrating that cytoskeletal rearrangement and organization are required for activation of Raf, even when it is targeted to the membrane. These data suggest that initial and sustained phases of integrin-mediated Raf activation are governed by distinct mechanisms that require Raf membrane localization and possibly PKC activity, respectively, and that integrin-mediated adhesion may regulate a membrane- or cytoskeleton-associated factor(s) responsible for Raf activation.

1732

MUTATION OF ANTI-ANGIOGENIC SEQUENCES IN THE TYPE I REPEATS OF THROMBOSPONDIN-1

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The extracellular matrix protein Thrombospondin-1 (TSP1) plays an important role in tumor metastasis by modulating angiogenesis, tumor cell motility, proliferation and proteolytic activities. Overexpression of TSP1 in breast carcinoma cells suppresses tumor growth in nude mice. It has been shown that synthetic peptides from the putative heparin binding motif in the type I repeats of TSP1 with a consensus sequence of W-S-X-W can reproduce the effects of TSP1 on the proliferation and motility of endothelial cells. We mutated the central Trp in each type I repeat to inactivate both WSXW motifs. MDA435 cells stably transfected with TSP1 with disruptions of second type I repeat (W441A) produced larger tumors than the wild type TSP1 transfectants. We have studied the effects of type I repeat mutants of thrombospondin on cell proliferation of transiently transfected MDA435 breast carcinoma cells and bovine aortic endothelial cells. In both the cell lines, wild type TSP1 expression inhibited the proliferation of the cells as measured by ^{3}H-thymidine uptake. The type I repeat mutants W385A and W498G showed similar inhibitions on cell proliferation. However, the mutant F432A which inactivates the TGF-β activation sequence KRFK was toxic to the cells expressing it. Thus the second type I repeat of TSP1 plays a more important role than the first and third repeats in the antiproliferative activity of TSP1.

Appendix K: Tables

Table 1. List of Thrombospondin Mutants

Mutant	Comments
THBS W385A	Type I repeat 1 mutation of central Trp required for heparin binding to synthetic peptides
THBS W441A	Type I repeat 2 mutation of essential central Trp residue
THBS W498G	Type I repeat 3 mutation of essential central Trp residue
THBS F432A	TGF beta activation sequence mutant

Table 2. Analysis of GFP Co-expression In Transient Transfectants

MDA435 cells were transiently transfected with 10 µg of wild type or F432A mutant DNA and 2.5 µg of the pGreen Lantern vector as described in Methods. Transfected cells were plated into 6-well tissue culture plates, and at 24 and 48 hours cells were counted under phase contrast (Total) or fluorescent (GFP +ve) light in three different fields of view.

Time	Wild Type			F432A Mutant		
	Total	GFP +ve	Percent	Total	GFP +ve	Percent
24 hr	93	12	12.9	43	3	6.9
	138	21	15.2	54	3	5.5
	47	7	14.8	95	6	6.3
		Average	14.3		Average	6.2
48 hr	Total	GFP +ve	Percent	Total	GFP +ve	Percent
	135	12	8.8	40	1	2.5
	126	13	10.3	65	0	0
	39	4	10.2	109	7	6.4
		Average	9.8		Average	2.9

Table 3: Effects of wild type and mutant TSP1s on proliferation of BAE cells.
Effects of the different transiently expressed proteins on proliferation of BAE cells was measured as ^{3}H-thymidine incorporation. Varying amounts of THBS wild type expression vector (WT) and 15-20 μg of the KRFK mutant (F432A), the first type I repeat mutant (W385A), the second type I repeat mutant (W441A), the third type I repeat mutant (W498G) or the truncation mutant (WG1a) were used for transfection and thymidine incorporation was measured as mentioned in the methods section. The transfection efficiencies are represented as β-galactosidase/μg total protein. The numbers in parentheses indicate number of samples assayed per group.

Sample	^{3}H-thymidine uptake (% Control ± SEM)	Transfection Efficiency as mU β-gal/μg protein (% Control ± SEM)
WT - 6 μg	90.41 ± 14.16	118.20 ± 13.61 (4)
WT - 12 μg	72.82 ± 9.01	124.19 ± 18.71 (4)
WT - 18 μg	48.34 ± 13.41	122.16 ± 41.99 (4)
F432A -18 μg	64.30 ± 19.35	143.25 ± 15.60 (2)
W385A - 15μg	128.01 ± 40.75	84.43 ± 17.59 (3)
W441A -19.5 μg	53.88 ± 8.33	127.36 ± 18.99 (5)
W498G - 16.5 μg	66.31 ± 13.49	129.15 ± 57.47 (3)
WG1a - 16.5 μg	19.06 ± 7.45	259.60 ± 104.35(2)

FIGURE 1

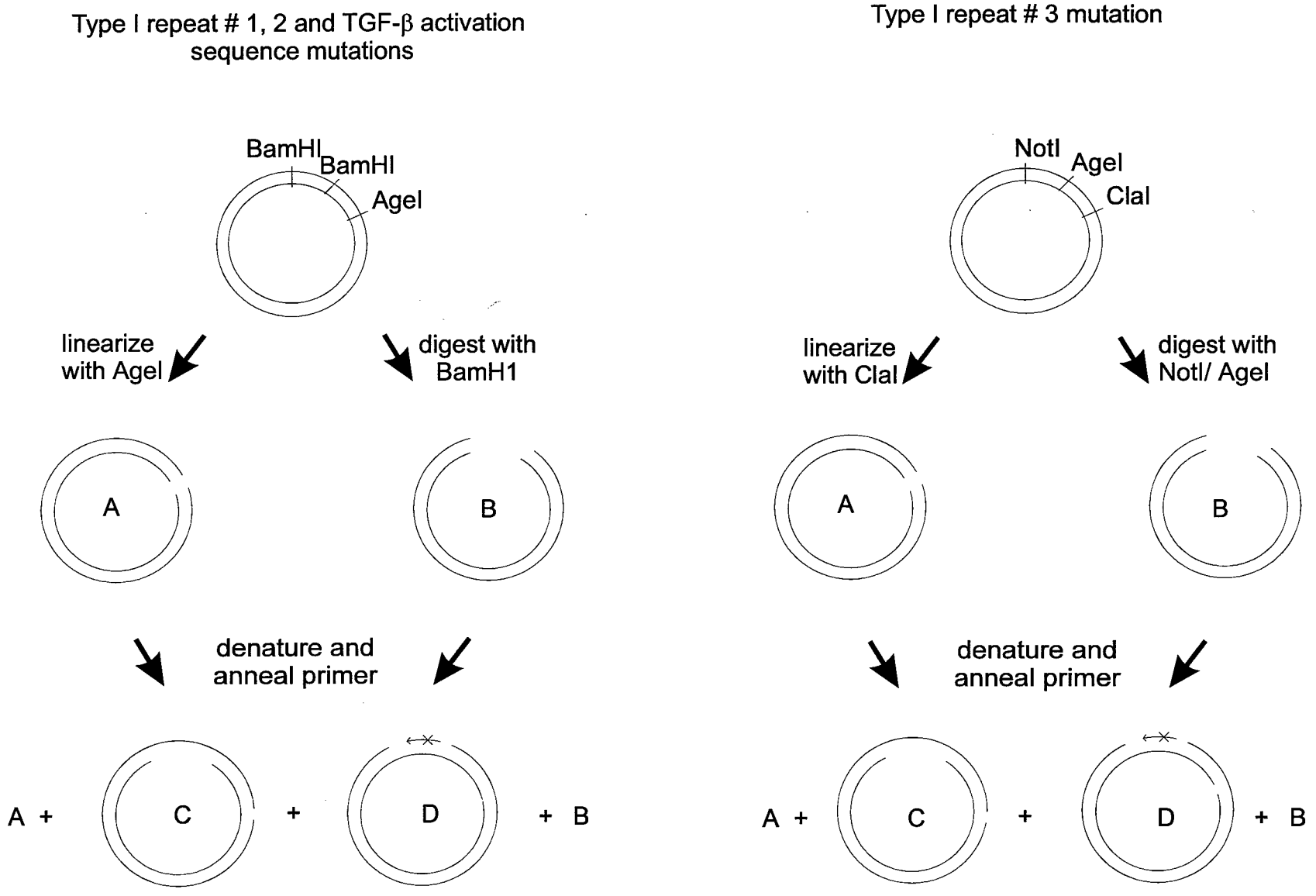


FIGURE 2

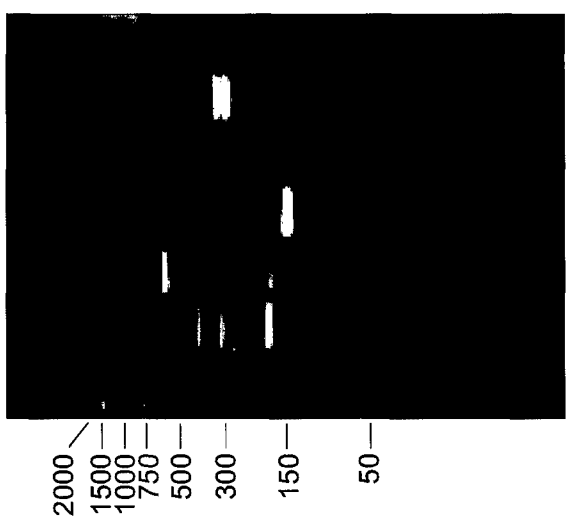


FIGURE 3

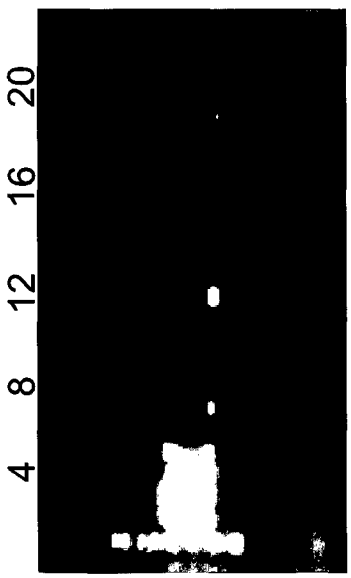


FIGURE 4

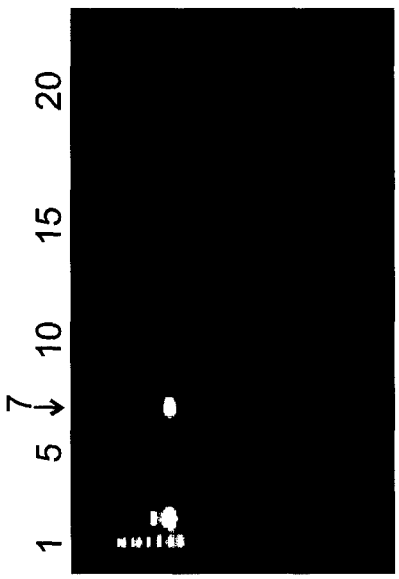
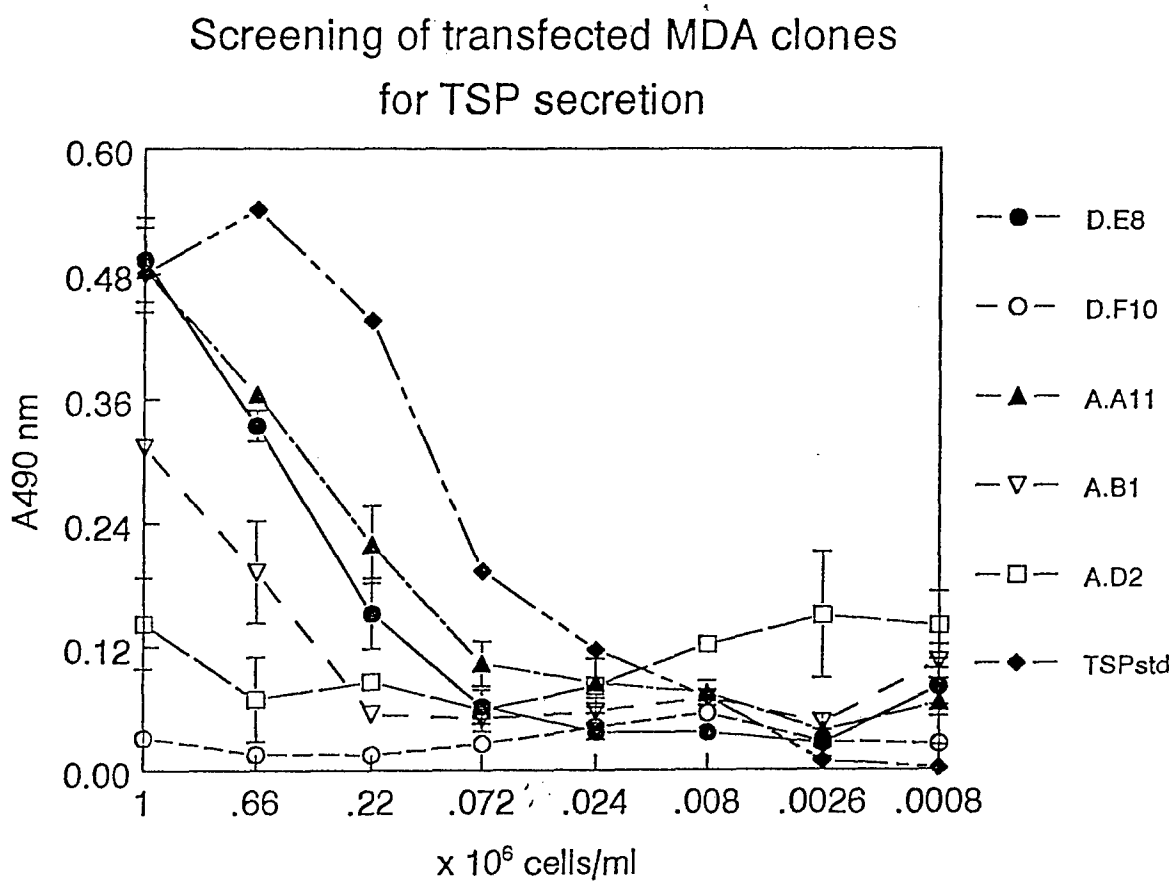
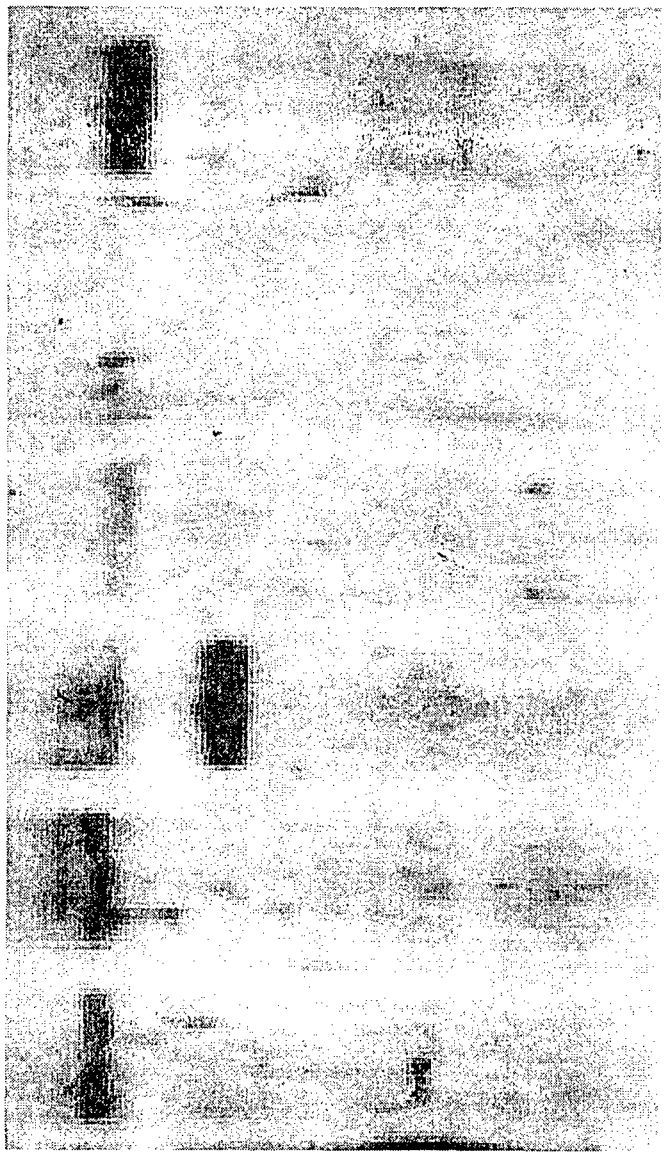


FIGURE 5


Screening of transfected MDA clones
for TSP secretion
A490 nm
0.60
0.48
0.36
0.24
0.12
0.00
1
.66
.22
.072
.024
.008
.0026
.0008
x 10^6 cells/ml
D.E8
D.F10
A.A11
A.B1
A.D2
TSPstd



TSP(FA) A.E10

TSP

TSP(WA) D.C5

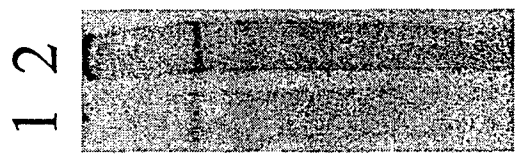
TSP(WA) A.A11

TSP(WA) D.F11

TSP(WA) E.B4

FIGURE 6

FIGURE 7



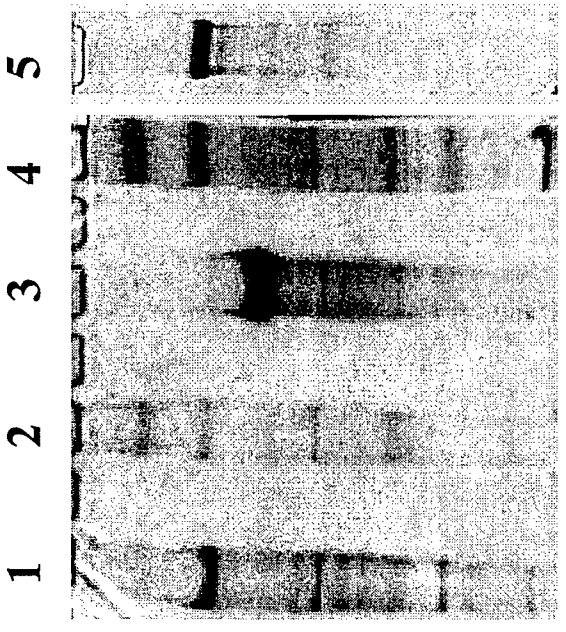


FIGURE 8

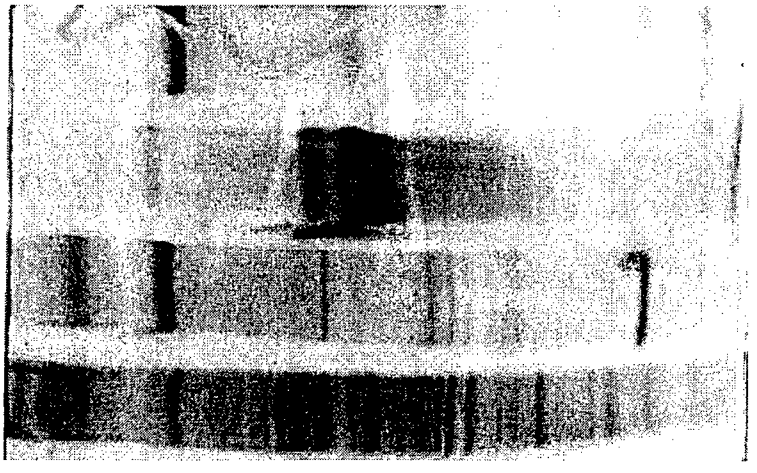


FIGURE 9

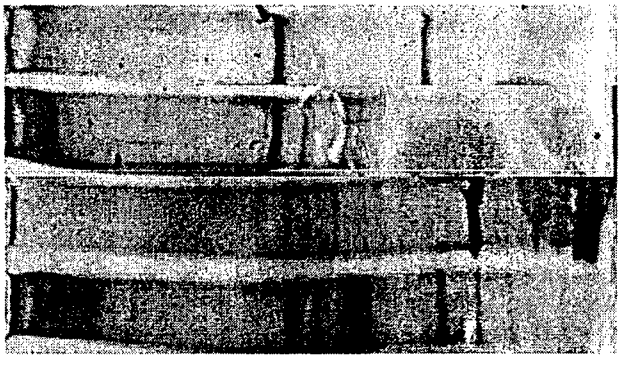


FIGURE 10

FIGURE 11

1 2 3 4

FIGURE 12

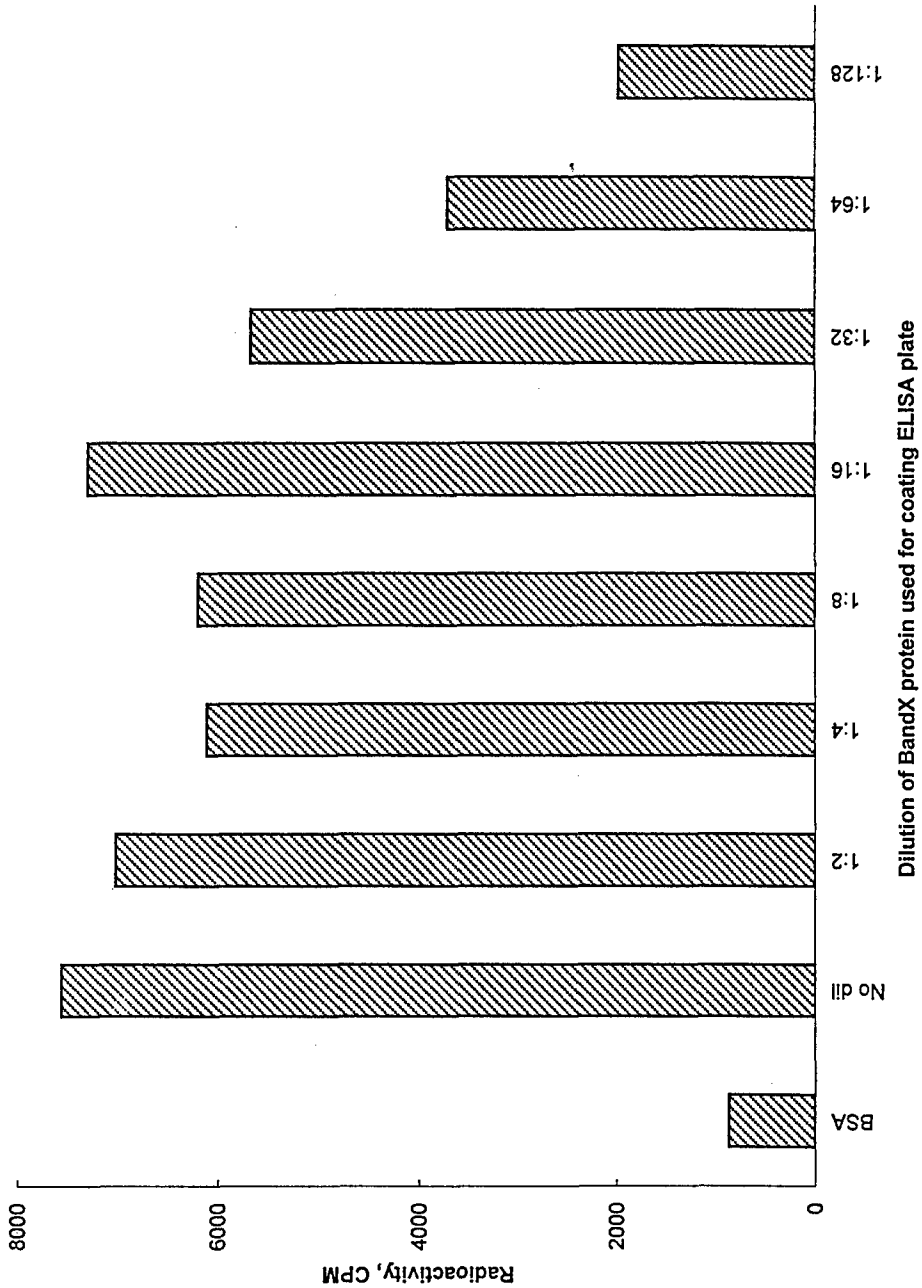

Radioactivity, CPM
0
2000
4000
6000
8000
BSA
No dil
1:2
1:4
1:8
1:16
1:32
1:64
1:128
Dilution of BandX protein used for coating ELISA plate

FIGURE 13

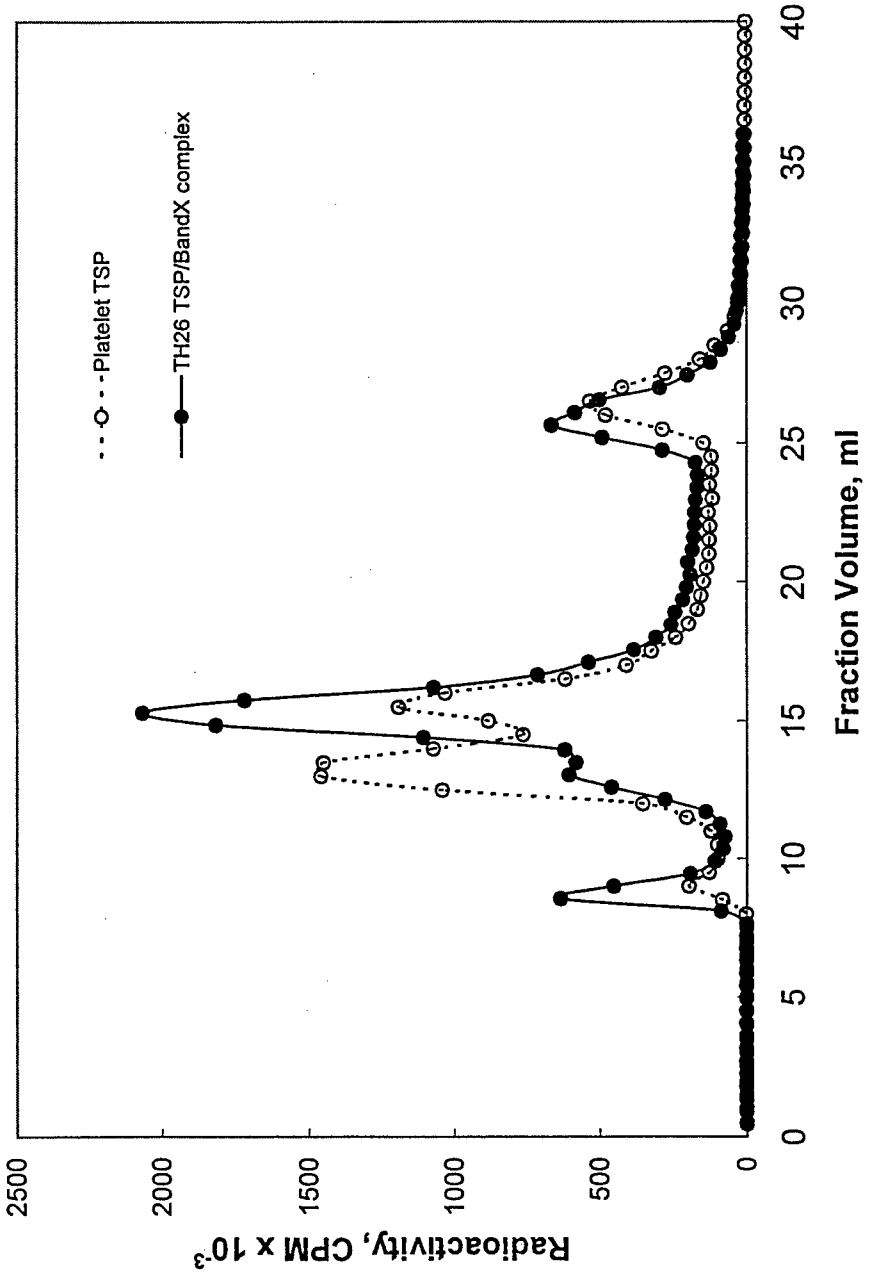
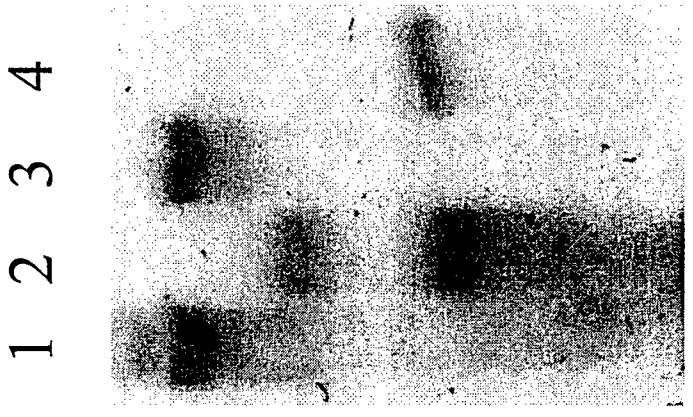

Platelet TSP
TH26 TSP/BandX complex
Radioactivity, CPM x 10-3
2500
2000
1500
1000
500
0
0
5
10
15
20
25
30
35
40
Fraction Volume, ml

FIGURE 14



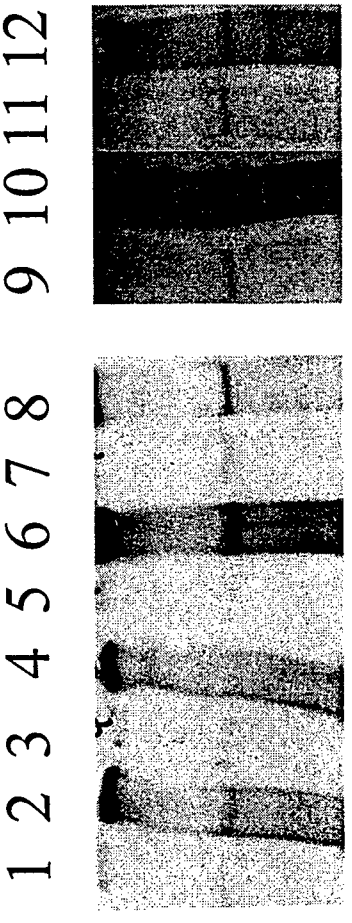


FIGURE 15

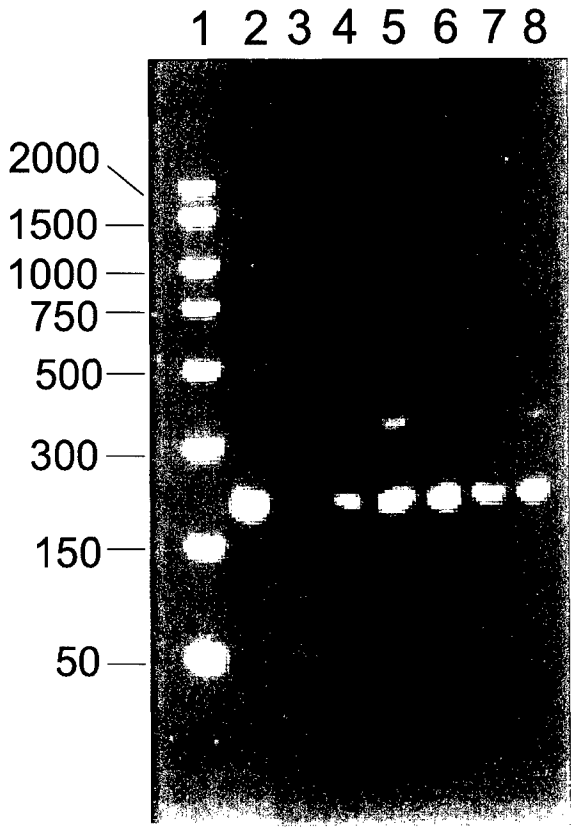
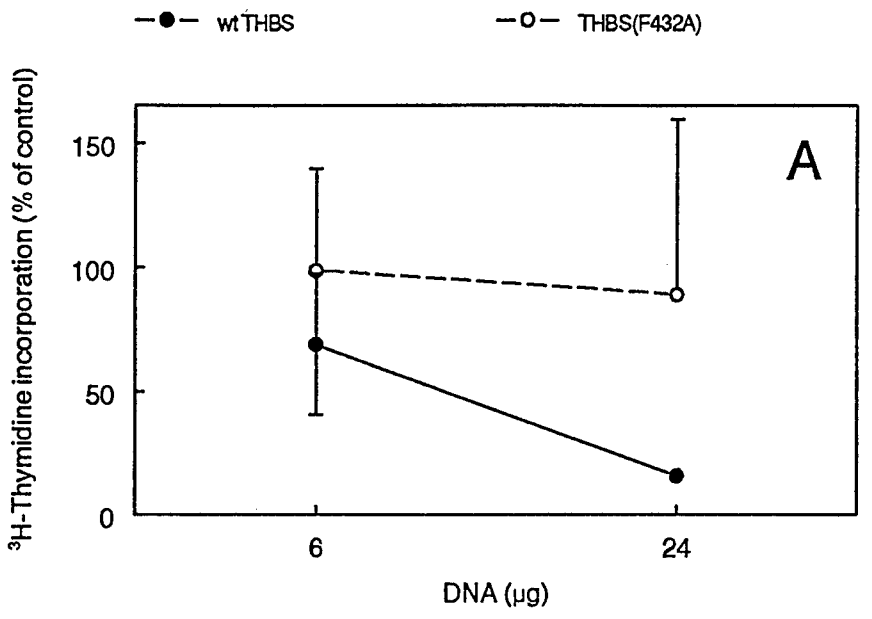


FIGURE 16


wt THBS
THBS(F432A)
^{3}H-Thymidine incorporation (% of control)
150
100
50
0
6
24
DNA (µg)
A

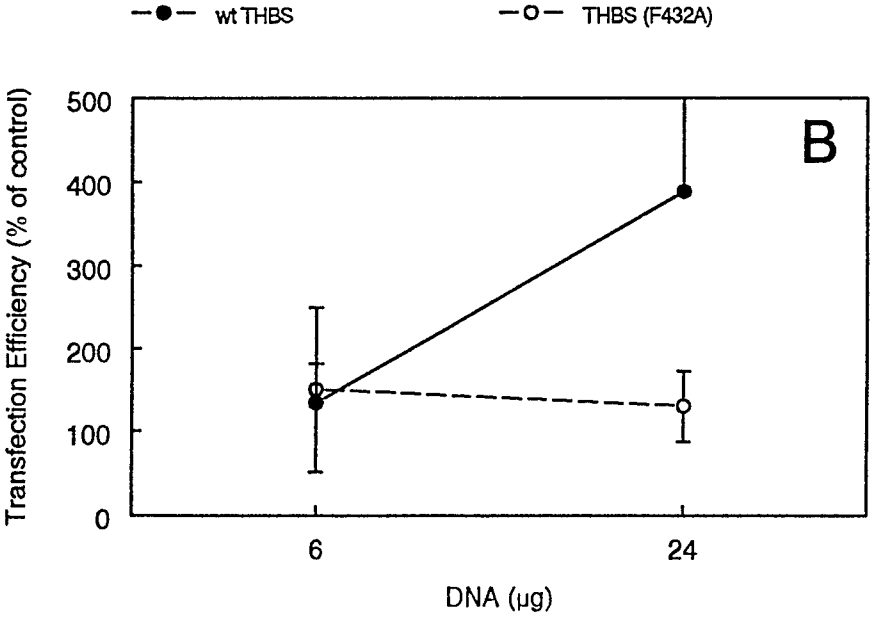

wt THBS
THBS (F432A)
Transfection Efficiency (% of control)
500
400
300
200
100
0
6
24
DNA (µg)
B

FIGURE 17

FIGURE 18

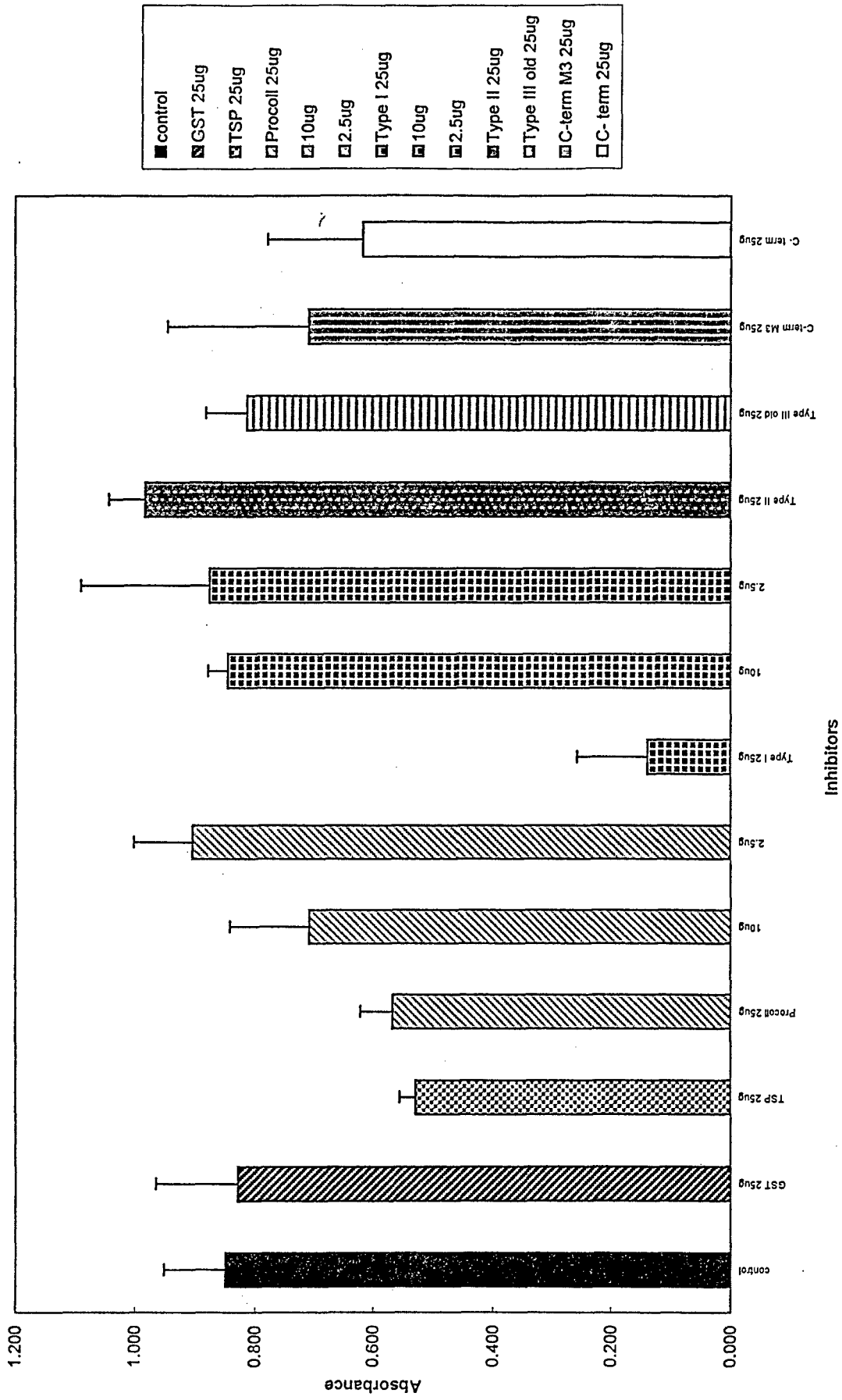

Proliferation of BAE Cells in the presence of recombinant TSP proteins
control
GST 25ug
TSP 25ug
Procoll 25ug
10ug
2.5ug
Type I 25ug
10ug
2.5ug
Type II 25ug
Type III old 25ug
C-term M3 25ug
C- term 25ug
Absorbance
1.200
1.000
0.800
0.600
0.400
0.200
0.000
control
GST 25ug
TSP 25ug
Procoll 25ug
10ug
2.5ug
Type I 25ug
10ug
2.5ug
Type II 25ug
Type III old 25ug
C-term M3 25ug
C- term 25ug
Inhibitors

FIGURE 19

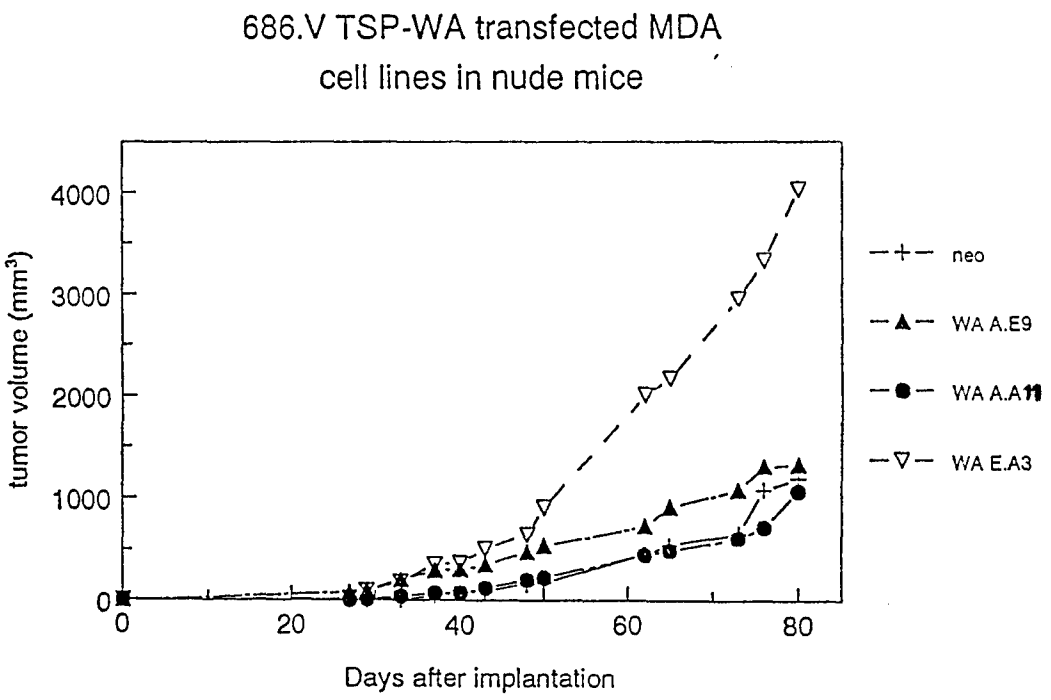
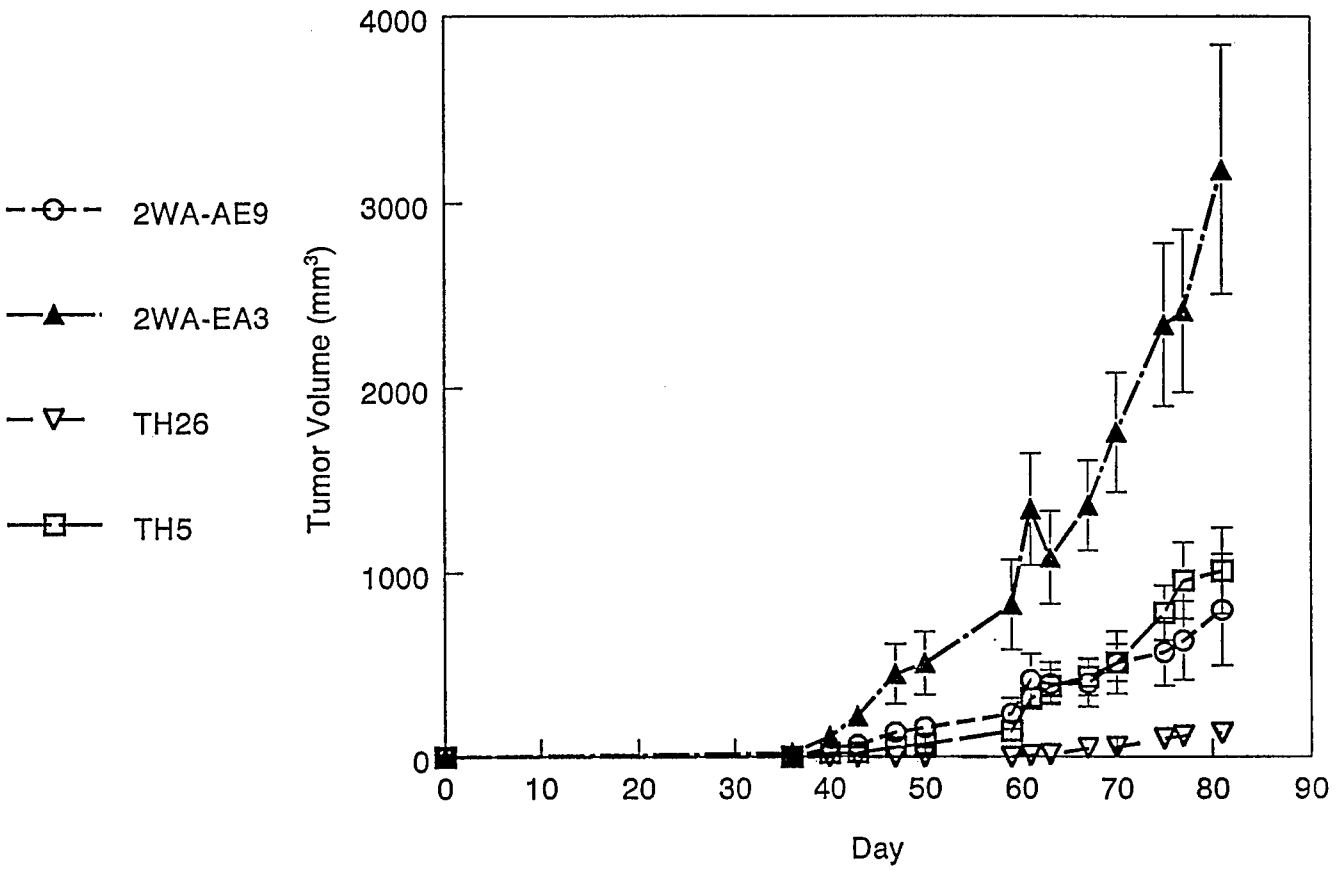

686.V TSP-WA transfected MDA
cell lines in nude mice
tumor volume (mm³)
4000
3000
2000
1000
0
0
20
40
60
80
Days after implantation
neo
WA A.E9
WA A.A11
WA E.A3

FIGURE 20


Tumor Progression in Beige Mice 96-02
MDA-WT and 2WA Mutants
2WA-AE9
2WA-EA3
TH26
TH5
Tumor Volume (mm3)
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
Day

Appendix L: Figures and figure legends

FIGURE LEGENDS

Figure 1: Strategy of construction of mutant DNAs. A, B, C and D represent the different forms of the double stranded expression vector pCMVTHBS containing the full length cDNA formed by reannealing the indicated fragments.

Figure 2: Touchdown PCR of mutant fragments of THBS. Lane 1 is the size marker. Lanes 2, 4 and 6 are Touchdown products of Type I repeat 2, TGF-beta activation sequence and Type I repeat 1 mutant fragments respectively. Lanes 3, 5 and 7 are controls showing lack of products from the corresponding wild type templates.

Figure 3 : Screening of bacterial colonies for THBS W441A mutation by Touchdown PCR. Lane 1 is the size marker. Lanes 4 and 5 are mutant and wild type PCR fragments. Lanes 7 - 20 are bacterial colonies amplified by Touchdown PCR. DNA from the colony in lane 12 was positive for the mutation.

Figure 4 : Screening of bacterial colonies for THBS F432A mutation. Lane 1 is the size marker. Lane 2 and 3 are mutant and wild type PCR fragments respectively amplified by Touchdown PCR. Lanes 5 - 20 are bacterial colonies screened for the mutation. DNA from the colony in lane 7 was positive for the mutation.

Figure 5: ELISA assay for screening cloned MDA435 transfectants for expression of TSP1 in transfected clones

Figure 6: Western blot analysis of recombinant TSP1 expression by transfected MDA cell clones.

Figure 7: Immunoprecipitation of myc-his tagged TSP1: Myc-his tagged full-length THBS construct in pCMVneoBam vector was used to transiently transfect MDA435 cells and 24 hr post transfection the cells were labeled with 100 μCi of [^{35}S] methionine for 2 hr. The spent medium (lane 1) and cell lysate (lane 2) were immunoprecipitated with anti-TSP1 antibody as described in the 'Methods' section.

Figure 8: Electrophoretic analysis of TSP1 purified by heparin affinity chromatography. Electrophoresis was carried out as described under 'Methods' using thrombospondin purified from conditioned medium derived from untransfected MDA cells (lane 1), MDA cells transfected with 2WA-A.E9 mutant TSP1 (lane 2), TH50 mutant TSP1 (lane 3) and TH26 wild type TSP1 (lane 4). The gel was stained using Coomassie blue. The electrophoretic pattern of platelet thrombospondin is shown in lane 5.

Figure 9: Purification of TH26-TSP1 on heparin affinity chromatography. Heparin affinity

chromatography was carried out as described under 'Methods'. Lane 1 contains the conditioned medium used as the source for purification. Sample eluted using 0.65 M NaCl contained TSP1 band and the high molecular weight protein band-X (Lane 2). A pre-elution step with 0.35 M NaCl did not elute TSP1 or band-X (Lane 3). Platelet TSP1 run in the same gel is also shown (Lane 4).

Figure 10: Proteolytic digestion of band-X protein. Band-X protein eluted from heparin affinity chromatography was digested with trypsin or thrombin. Lanes 1 and 3 contain the starting material for the proteolytic digestion. The protease reaction was carried out as described under 'Methods' the proteolytic digests are shown in lane 2 (trypsin digest) and lane 4 (thrombin digest).

Figure 11: Western blotting analysis of thrombospondin. Heparin affinity chromatography purified thrombospondin from untransfected MDA (lane 1), TH26 wild type thrombospondin-transfected MDA (lane 2), 2WA- mutant-transfected MDA (lane 3), and thrombospondin purified from human platelets (lane 4) were run in a 4-15 % gradient gel under reducing condition. The proteins were then transferred to PVDF membrane and probed with anti-thrombospondin polyclonal antibody and the bands were visualized by ECL method.

Figure 12: Assay of thrombospondin interaction with band-X. ELISA plate was coated with different dilutions of band-X protein-purified by heparin affinity chromatography. Fixed amount of ^{125}I-labeled thrombospondin was used for binding (see Methods). The unbound material was rinsed out and the wells were removed from the plate and counted in a gamma counter. The data presented here represent the mean value of duplicate samples.

Figure 13: Gel filtration profile of TSP1/band-X complex compared to platelet TSP1. ^{125}I-labeled samples were used for gel filtration on Sephacryl S-500. Fixed volume fractions were collected and the radioactivity was measured in a gamma counter.

Figure 14: Agarose gel electrophoresis. Agarose gel (3%) was used to resolve TSP1 and TSP/band-X complex. TSP1 and band-X mixture under reducing and non-reducing conditions is shown in lane 1 and lane 2 respectively. Lanes 3 and 4 contain platelet TSP1 under reducing and non-reducing conditions respectively.

Figure 15: Synthesis and secretion of TSP1 mutants: MDA435 cells grown in 10 % fetal bovine serum were electroporated with 10 µg each of pCMVneoBam vector (lanes 1- 4), THBS wild type construct (lanes 5 - 8) or F432A mutant construct (lanes 9 - 12). 24 hr post transfection cells were pulse-labeled with 100 µCi of [^{35}S] methionine for 30 min and subsequently chased for 60 min (lanes 1, 2, 5, 6, 9, and 10) or 180 min (lanes 3, 4, 7, 8, 11 and 12). Spent media (lanes 1, 3, 5, 7, 9 and 11) and cell lysates (lanes 2, 4, 6, 8, 10 and 12) were immunoprecipitated with anti-TSP1 antibody as described in the 'Methods' section.

Figure 16: RT-PCR of mammary fat pad tumors from mice injected with THBS W441A transfectant clones. Lane 1 is the size marker. Lane 2 is the RT-PCR product of wild-type THBS

cDNA in the expression vector. Lanes 3, 4 and 5 are products from mouse tumors expressing the THBS W441A - A.A11 clone and lanes 6, 7 and 8 are products from tumors expressing the E.A3 clone.

Figure 17: Dose response for inhibiting proliferation of MDA435 breast carcinoma cells by expression of wild type TSP1 or TSP1 with mutation in the TGFβ activation sequence (KRFK). (Panel A) ^{3}H-thymidine incorporation by MDA435 cells transiently transfected with varying amounts of THBS wild type expression vector (WT) and the KRFK mutant (F432A) were measured as described in the methods section. (Panel B) The transfection efficiencies were determined by co-transfection with a constant amount of β-galactosidase vector. Efficiency was calculated as β-galactosidase/ μg total protein and is presented as a percent of control transfections without addition of the THBS vectors.

Figure 18: Proliferation Assay using bovine aortic endothelial cells with recombinant fragments of different domains of TSP1. The amounts indicated are μg/ml. Values are averages of triplicate wells. The amino acid residues of TSP1 represented in each of the recombinant fragments used are - Procollagen, 278 - 355; Type I repeat, 385 -522; Type II repeat, 559 - 669; Type III repeat, 784 - 932; COOH-terminal (M3), 877 - 1152; COOH-terminal, 933 -1152.

Figure 19: Growth curves for MDA435(W441A) transfectants in athymic nude mice.

Figure 20: Growth of stably transfected MDA435 breast carcinoma cells implanted in the mammary fat pads of NIH-III Beige nude XID mice. Tumor volumes were determined by external caliper measurements at the indicated times after implantation of MDA435 control transfectant (□), clone TH-26 over expressing wild type thrombospondin (▽), clone AE9 expressing W441A thrombospondin (○), or clone EA3 expressing W441A thrombospondin (▲). Results are mean ± SD, n = 10.

Appendix M

BIBLIOGRAPHY

Peer reviewed publications

1. Roberts, D. D.: Regulation of tumor growth and metastasis by thrombospondin-1. *FASEB J.* 10:1183-1191, 1996.

2. Guo, N., Krutzsch, H.C., Inman, J.K., and Roberts, D.D.: Thrombospondin-1 and type I repeat peptides of thrombospondin-1 specifically induce apoptosis of endothelial cells. *Cancer Res.* 57:1735-1742, 1997.

3. Guo, N., Krutzsch, H.C., Inman, J.K., and Roberts, D.D.: Anti-proliferative and anti-tumor activities of D-reverse peptide mimetics derived from the second type-I repeat of thrombospondin-1. *J. Peptide Res.* 50:210-221, 1997.

4. Chandrasekaran, S., Guo, N., Rodrigues, R. G., Kaiser, J., and Roberts, D. D.: Pro-adhesive and chemotactic activities of thrombospondin-1 for breast carcinoma cells are mediated by α3β1 integrin and regulated by insulin-like growth factor and CD98. *J. Biol. Chem.* 274:11408-11416, 1999

5. Iruela-Arispe, M. L., Lombardo, M., Krutzsch, H. C., Lawler, J., and Roberts, D. D.: Inhibition of angiogenesis by thrombospondin-1 is mediated by two independent regions of the type 1 repeats. *Circulation* 100: 1423-1431, 1999.

6. Krutzsch, H. C., Choe, B., Sipes, J. M., Guo, N., and Roberts, D. D.: Identification of an α3β1 integrin recognition sequence in thrombospondin-1. *J. Biol. Chem.* 274: 24080-24086, 1999.

7. Shafiee, A., Penn, J. K., Krutzsch, H. C., Inman, J. K., Roberts, D. D., and Blake, D. A.: Peptides derived from the type 1 repeats of thrombospondin-1 inhibit angiogenesis in a bovine retinal explant assay and a rat model of retinopathy of prematurity. (submitted)

8. Chandrasekaran, L., He, C-Z., Krutzsch, H. C., Iruela-Arispe, M. L., and Roberts, D. D.: Modulation of endothelial cell behavior and angiogenesis by an α3β1 integrin-binding peptide from thrombospondin-1. (Submitted)

Abstracts

1. Guo, N., Krutzsch, H. C., Inman, J. K., and Roberts, D. D. Thrombospondin type 1 repeats specifically induce apoptosis of endothelial cells. The thrombospondin gene family and its functional relatives tenascins, osteopontin, and SPARC. Seattle, WA, 1996.

2. Roberts, D. D., Guo, N., Chandrasekara, L., Chandrasekaran, S., Inman, J. K., Krutzsch, H. C. Rolke of type 1 repeats in inhibition of breast carcinoma growth by thrombospondin-1. Era of Hope meeting, 1997.

3. Chandrasekaran, L., and Roberts, D. D. Mutation of anti-angiogenic sequences in the type 1 repeats of thrombospondin-1. Molec. Biol. Cell 9:298a, 1998.

Appendix N

Personnel

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Joe Tseng, B.S.

Rui Rodrigues, B.S.

Heba Al-Barazi, Ph.D.

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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be changed to "Approved for public release; distribution unlimited"

Grant Number	Accession Document Number
DAMD17-94-J-4147	ADB221256
DAMD17-93-C-3098	ADB231640
DAMD17-94-J-4203	ADB221482
DAMD17-94-J-4245	ADB219584
DAMD17-94-J-4245	ADB233368
DAMD17-94-J-4191	ADB259074
DAMD17-94-J-4191	ADB248915
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DAMD17-96-1-6092	ADB239339
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DAMD17-95-C-5078	ADB232058
DAMD17-95-C-5078	ADB232057
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DAMD17-95-C-5078	ADB253038
DAMD17-95-C-5078	ADB261561
DAMD17-94-J-4433	ADB221274
DAMD17-94-J-4433	ADB236087
DAMD17-94-J-4433	ADB254499
DAMD17-94-J-4413	ADB232293
DAMD17-94-J-4413	ADB240900

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